REGULATION OF PROTEIN SYNTHESIS BY ABSCISIC ACID AND PHASEIC ACID IN BARLEY ALEURONE LAYERS

by

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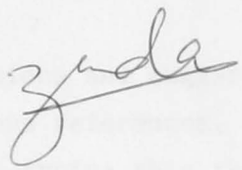
STATEMENT

This thesis has not previously been submitted for an academic award at this or any other University, and is the original work of the author, except where due reference is made in the text.



(Zuraida Ariffin).

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ABA-GE	Abscisic acid-glucose ester
ADH	Alcohol dehydrogenase
ASI	α-amylase/subtilisin inhibitor
BSA	Bovine Serum Albumin
cpm	Counts per minute
DAE	Days after emergence
DMSO	Dimethyl sulfoxide
DPA	Dihydrophaseic acid
GA_3	Gibberellic acid
HS	Heat shock
IAA	Indole acetic acid
M_r	Relative mobility
PA	Phaseic acid
PEG	Polyethylene glycol
PMSF	Phenylmethyl-sulfonyl fluoride
PVP	Polyvinyl-pyrrolidone
SDS	Sodium Dodecyl Sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TES	N-*tris*(hydroxymethyl)methyl-2-aminoethane-sulfonic acid
WGA	Wheat germ agglutinin

ABSTRACT

ABA induces the synthesis of several polypeptides in barley aleurone layers and suppresses the synthesis of GA_3-promoted α-amylase. Synthesis of 'ABA polypeptides' with differing induction kinetics and duration of synthesis, requires the presence of a threshold concentration of ABA between 0.1 μM and 0.3 μM. The induction of "ABA proteins" by 1 μM ABA is negated by the simultaneous addition of GA_3 as evident from comparison of polypeptide profiles for aleurone layers incubated in 1 μM ABA (only) and in 1 μM ABA+ 0.05 μM GA_3. The activity of PA is virtually the same as that of ABA in promoting 'ABA polypeptides' and suppressing synthesis of α-amylase. Most PA- and ABA-induced polypeptides remain in solution after heat treatment, whereas the majority of 'control' proteins are precipitated. A low proportion of particular 'ABA polypeptides' are secreted.

Cell-free translation studies demonstrated that ABA-induced changes in protein synthesis are correlated with changes in mRNA abundance. PA also regulates abundance of the mRNA species which are induced by ABA. Some of the *in vitro* products whose syntheses are programmed by RNA from ABA- or PA-treated aleurone layers are also retained in solution following heat treatment, as are the *in vivo* PA- and ABA-induced polypeptides. By contrast, virtually all of the *in vivo* and *in vitro* polypeptides of untreated aleurone layers precipitate when subjected to heat treatment.

In the wheat-germ system, examination of non-heated proteins indicate that *in vitro* polypeptides only sometimes have *in vivo* co-migrating counterparts. In the rabbit reticulocyte system, comparison

of heated proteins indicate that *in vitro* products (with the exception of an $M_r \sim 31,000$ polypeptide) co-migrate with an *in vivo* polypeptide. However, several abundant *in vivo* polypeptides do not match up with *in vitro* polypeptides. A precursor-product relationship is suggested for an $M_r \sim 31,000$ (*in vitro*) and an $M_r \sim 30,000$ (*in vivo*) polypeptide, which bears similarity with one of the polypeptides secreted by aleurone layers.

Seven ABA-induced cDNA clones were used as probes in hybridization analyses and two of these (pHV A34 and pHV A39) were selected for more detailed studies. Specific ABA-induced mRNAs are promoted to maximal abundance at about 24 h in aleurone layers treated with 25 µM ABA. Incubation of aleurone layers in PA also results in the induction of these specific mRNAs to the same level as that induced by an equal concentration of ABA.

Aleurone layers of developing barley grains are seen to display increased levels of ABA-specific mRNAs. Since it has been observed that endogenous ABA levels increase during development in other cultivars of barley other than Himalaya, the appearance of these ABA-specific mRNAs may occur in response to the increased endogenous ABA in the aleurone layers of developing barley grains.

The nucleotide sequence of pHV A34 and its deduced amino acid sequence do not show significant homology with sequences of any known proteins.

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INTRODUCTION

History

Abscisic acid (ABA) was first detected as inhibitor-β by researchers who were studying the effect of auxins (reviewed by Addicott and Carns, 1983). Following the discovery of auxin in the 1930s, techniques were developed to isolate plant hormones. The standard technique used in auxin physiology was the extraction of plant tissue with ether followed by application of the concentrated extract to *Avena* coleoptiles, and measurement of subsequent growth. In the late 1940s, paper chromatographic methods were developed to fractionate samples containing small amounts of active substances. When the acid fraction of ether extracts of a variety of plant materials were paper chromatographed, a certain spot contained an inhibitor (of *Avena* coleoptile and pea root extension) and this substance was called inhibitor-β (Bennet-Clark and Kefford, 1953). In 1967 at an international meeting in Ottawa, a plenary session was held to discuss a name for three identical substances which had been discovered through several independent lines of investigation. These substances were cotton abscisin II, sycamore dormin, and lupin abscission accelerator. Abscisin II was purified from young cotton fruit and it was named in recognition of its abscission-accelerating property when applied to petiole stumps of cotton seedling explants (Ohkuma *et al.*, 1963). These workers also showed that abscission-acceleration resulting from the application of 1.0 and 0.1 µg of abscisin II was greater than can be obtained from maximum abscission-accelerating amounts of gibberellin A_3(GA_3). Phillips and Wareing (1958) showed that seasonal changes in the inhibitor content

of sycamore were correlated with dormancy of the shoot apexes. The inhibitor, named dormin, was isolated by paper chromatography and its activity measured using several bioassays. Van Steveninck (1959) utilized the yellow lupin and determined that developing fruit produced a substance that induced the abscission of flowers and young fruit higher up the inflorescence. The lupin abscission accelerator also inhibited wheat coleoptile extension and had the chromatographic properties of inhibitor-β. Although abscisin II was the first to be isolated and characterised, the name abscisic acid, using the form of "-ic acid" was chosen to avoid problems in naming analogues and derivatives.

Significance of Abscisic Acid in Plants

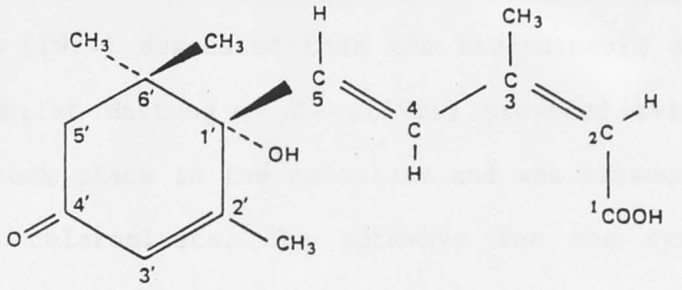
Abscisic acid is found in all higher plants which have been examined. In whole plants, ABA may be able to confer resistance to stresses brought about by a changing environment, such as drought, salinity, high temperatures, cold and waterlogging. Plant responses to ABA may be fast or slow. In corn, stomatal closure is observed within three minutes when ABA is applied to the cut base of a leaf (Kriedemann *et al.*, 1972). However, in instances where a response to ABA involves changes in protein synthesis, as for suppression of α-amylase synthesis, the change is not observed until 4 to 8 hours (Ho, 1979). The multi-faceted effects of endogenous ABA are difficult to define due to our inability to isolate different types of environmental stresses: for instance, application of heat stress (37°C air stream) to a leaf may be registered as water stress in the plant due to the increased rate of evapotranspiration in the leaf (Goldbach *et al.*, 1977).

The involvement of ABA in the adaptation of plants to environmental stresses was initially revealed by the correlation between ABA levels and the onset of stress. However, there are also situations when this correlation is not observed, due to changes in other hormones which are antagonistic to ABA. Many plant processes appear to be controlled by interacting factors; for example, ABA induces stomatal closure whereas IAA promotes stomatal opening (Eamus, 1986). It has also been documented that maintenance of status quo within plants requires fine tuning of ABA synthesis and degradation, so as to increase ABA during stress and to rapidly metabolise ABA to PA (which may still be active), dihydrophaseic acid and other metabolites when favourable conditions prevail for growth and metabolism (Pierce and Raschke, 1981).

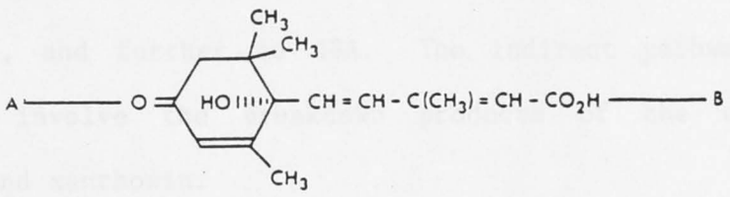
The importance of ABA in plants is also evident in, for instance, the viviparous mutants of maize which germinate prematurely while still on the cob, due to low endogenous levels of ABA (Wilson *et al.*, 1973).

Structure of Abscisic Acid

The chemical structure (Fig. 1) and stereochemistry of abscisin II (abscisic acid) were determined by Ohkuma *et al.* (1965). The ABA molecule (molecular weight 264) is asymmetrical about carbon 1' and therefore possesses optical activity. Chemical synthesis yielded the racemic compound (Cornforth *et al.*, 1965) which is a 1:1 mixture of (+)- and (-)- ABA. In a projection of the structural formula in Fig. 1(b), it can be seen that the features which give ABA its optical activity (along the axis of symmetry A-B) are at positions C-6' (carries two methyl groups) and C-2' (carries a methyl group and there is a double bond).



a Structure of (+)-(*S*)-abscisic acid.



b Projection of (+)-abscisic acid

Figure 1.

In some bioassays, the two enantiomers are equally active, while in others (+)-ABA is more active than (-)-ABA (Sondheimer *et al.*, 1971). Both isomers are equally effective in the inhibition of gibberellin-induced synthesis of α-amylase (Sondheimer *et al.*, 1971), whereas (+)-ABA is markedly more effective in bringing about stomatal closure (Cummins and Sondheimer, 1973, Kriedemann *et al.*, 1972). The (-)-enantiomer of ABA seemed to have a low intrinsic activity in causing stomatal closure although this could be due to incomplete resolution of enantiomers (Kriedemann *et al.*, 1972) or a difference in uptake (Sondheimer *et al.*, 1971).

Biosynthesis, Metabolism and Compartmentalization of ABA

Milborrow (1974) suggested that the biosynthesis of ABA occurred in plastids whilst Hartung *et al.*,(1981) provided evidence that ABA biosynthesis took place in the cytoplasm and was subsequently compartmentalized in chloroplasts. The pathways for the synthesis of ABA have been the subject of a great deal of research and there is evidence for and against two possible pathways (Milborrow 1983, Burden and Taylor 1976, Milborrow 1974). The direct pathway is believed to start from mevalonate which is converted to a C15 intermediate (farnesyl pyrophosphate), and further to ABA. The indirect pathway has been suggested to involve the breakdown products of the carotenoids, violaxanthin and xanthoxin.

ABA is metabolised in the following manner: ABA → PA (phaseic acid) → DPA (dihydrophaseic acid) → polar metabolites and conjugates (Harrison and Walton, 1975; Dewdney and McWha, 1978; Dashek *et al.*, 1979; Pierce and Raschke, 1981; Uknes and Ho, 1984). Pierce and Raschke (1981) showed that the water status of a plant may affect the metabolism of ABA: dehydration caused the stimulation of ABA synthesis, rehydration caused a lowering in the rate of synthesis of ABA and stimulation of its conversion to PA. This suggests that ABA levels are somehow regulated within the plant by modulating the rates of synthesis and breakdown of ABA.

In barley, the conversion of ABA → PA → DPA was found to be tissue-specific (Dashek *et al.*, 1979; Uknes and Ho, 1984) occuring in the aleurone layer of the grain but not in the starchy endosperm, embryo or leaf. Creelman and Zeevaart (1984) showed that during the conversion of ABA to PA, one atom of ^{18}O was incorporated into PA suggesting that the enzyme involved was an oxygenase.

PA has been shown to be as active as ABA in the inhibition of α-amylase synthesis (Dashek *et al.*, 1979); it is able to bring about stomatal closure in various plant species (Sharkey and Raschke, 1980) and promotes abscission, although PA has only one-tenth of the activity of ABA in this function (Davis *et al.*, 1972). It is not known if the other metabolites of ABA, apart from PA, have any physiological effect. DPA (dihydrophaseic acid) was shown to be inactive in several bioassays on the inhibition of growth and germination (Dashek *et al.*, 1979) and stomatal closure (Sharkey and Raschke, 1980). Therefore, this is consistent with the hypothesis that ABA levels are partly modulated by breakdown of ABA, but probably not until the conversion has progressed as far as DPA.

Heilmann *et al.*, (1980) suggested that the difference in the concentration of ABA between the chloroplasts and other intracellular compartments can be explained by the weak acid property of ABA (pK 4.8), and that the undissociated form of ABA is able to move rapidly through the chloroplast membrane. The chloroplast has a relatively high pH (pH 7.5 in the dark, pH 8 in the light) compared with the cytoplasm which has a lower pH (∿7). The pH difference is increased during illumination, resulting in the accumulation of ABA within the stroma (Heilmann *et al.*, 1979; Cowan *et al.*, 1982). On the other hand, any factor that tends to reduce the pH of the stroma would be expected to increase the amount of ABA available. For instance, this may happen under water stress when it is possible that the stroma pH is reduced, resulting in the increased availability of ABA, via the free space (i.e. cell wall, xylem elements and tracheids) to the guard cells for enhancement

of stomatal closure (Cowan *et al.*, 1982). Calculations based on [^{14}C]-ABA uptake data indicated that the pH of chloroplasts was reduced under water stress imposed by hyperosmosis using sorbitol (Heilmann *et al.*, 1980) and this was confirmed by Schuldiner *et al.* (1972) using the 9-amino-acridine technique. However, it is not known if water stress imposed by a reduction in turgor pressure (e.g. through high transpiration rate in leaves) would also result in a lowered pH in the stroma of chloroplasts.

Bray and Zeevaart (1985) studied the localization of free ABA and the conjugate, ABA-glucose ester (ABA-GE) by dimethyl sulfoxide (DMSO) disruption of the plasmalemma and tonoplast (membrane surrounding the vacuole) under certain conditions. It was found that free ABA was located in a compartment not accessible to DMSO disruption, the possible location being chloroplasts. This was consistent with the data of Hartung *et al.* (1981). The observations on ABA-GE release by DMSO disruption, coupled with the analysis of ABA-GE concentration in isolated vacuoles enabled Bray and Zeevaart (1985) to conclude that ABA-GE was sequestered within vacuoles and that conjugation is a means whereby ABA is removed away from the active pool.

From the above, it appears likely that ABA is synthesised in the cytoplasm and compartmentalised within chloroplasts. The intracellular movement of endogenous ABA may be dependent upon the pH gradients within the cell (Heilmann *et al.*, 1979; Cowan *et al.*, 1982) and water stress causes higher levels of ABA to be released from the chloroplasts into the cytoplasm.

The Effect of ABA on Stomata

ABA plays an essential role in the control of plant water relations e.g. wilty mutants of tomato are not able to modulate water loss via stomatal closure due to low endogenous ABA levels within the plant (Imber and Tal, 1970; Tal and Imber, 1970). For experimental purposes, water stress may be induced by withholding water from root systems of intact plants, by rapid wilting of detached leaves in a stream of warm air or by osmotic means (polyethylene glycol (PEG), salt). Plants would have to cope with situations of water stress by sending a signal, in this case ABA, to the guard cells to control stomatal aperture to minimise water loss by transpiration and yet still allow gas exchange for respiration and photosynthesis.

ABA is one of the most important regulators of stomatal closure in the intact leaf. Although the role of ABA in promoting stomatal closure has been much studied, the mechanism by which this effect is achieved has not been firmly established.

Wright and Hiron (1969) showed that when excised wheat leaves were wilted under a stream of air (25°C), the levels of (+)-ABA increased forty-fold over those of controls. Goldbach and Goldbach (1977) showed that water stress imposed on intact barley plants by subjecting only the leaves to warm air (36°C) caused significant increases in ABA in all plant parts, especially the ears, and suggested that ABA synthesised in the leaves is transported to other parts of the plant. They did not detect ABA increases as high as those of Wright and Hiron (1969) and this was probably due to the difference in the systems used: presumably excised leaves accumulated higher levels of ABA due to the

absence of sinks that intact plants possess i.e. ABA synthesised in the leaves may be transported to the stems, ears and roots in intact plants. The effect of ABA on stomatal closure, and hence transpiration, was shown by making cellulose acetate imprints of silicone rubber impressions of wheat and barley leaves which were supplied with ABA through the transpiration stream (Mittelheuser and Van Steveninck, 1969). Photographs of these imprints showed that stomata of leaves in water were open (2-3.3 µm) while those of leaves placed in ABA (3.8 µM) solutions were closed, resulting in the transpiration rate of ABA-treated leaves being lower than those of controls.

The lag times for stomatal closure may vary between species (e.g. 3 minutes for corn, and 32 minutes in rose leaves) and the quickness or slowness of the response seen in excised leaves may be dependent upon a certain magnitude of increase in the endogenous ABA levels (Kriedemann *et al.*, 1972). However, the very rapid stomatal closure (3 minutes for corn leaf) may also be a reflection of the greater uptake of ABA via the transpiration stream i.e. when excised leaves are placed in a solution containing ABA.

CO_2 was shown to cause stomatal closure without any effect on the endogenous ABA concentrations in intact vine leaves (Loveys *et al.*, 1973). The invalidation of the idea that ABA effects stomatal closure due to side-effects such as inhibition of photosynthesis was established after it was shown that stomatal closure was observed much earlier than the inhibition of photosynthesis (Mittelheuser and Van Steveninck, 1971). On the basis of these findings, it was suggested that the most likely relationship between the effect of endogenous ABA in regulation of stomatal aperture and rates of photosynthesis was that stomatal closure

caused a reduction of gas exchange which ultimately led to inhibition of photosynthesis. However, more recent data has provided some evidence for a direct effect of ABA on the inhibition of photosynthesis (Dubbe *et al.*, 1978).

Raschke (1975) demonstrated the simultaneous requirement of CO_2 and ABA for stomatal closure in detached leaves of *Xanthium*. Wilson (1981), however, found that stomatal responses to applied ABA and CO_2 had a certain degree of interdependence in epidermal strips of *Commelina*: the stomata responded to ABA supplied in the incubation medium in the presence and virtual absence of CO_2, however the response was more obvious in high-CO_2 air. These contradictory observations could be due to differences in the systems (detached leaves versus epidermis) or the plant species used.

It has been known for a long time that ABA affects guard cells by causing a reduction in the turgor pressure within the guard cells, caused by the movement of potassium ions through the plasmalemma. Using microautoradiography and $^{86}Rb^+$ as a tracer for potassium ions, it was shown that in *Commelina* epidermis, $^{86}Rb^+$ was taken up largely into the guard cell region and that ABA affected both uptake and release of $^{86}Rb^+$ (Weyers and Hillman, 1980). However, epidermal strips may not provide a good system for the study of ionic fluxes if there is a high proportion of living epidermal cells (surrounding guard cells) which may cause redistribution of ions. The alternative system, used by Macrobbie (1981), was 'isolated' guard cells and her results confirmed those of Weyers and Hillman (1980) in showing that the effect of ABA resided mainly in the stimulation of ion effluxes through guard cells,

rather than on the inhibition of influxes. The higher turgor pressure in guard cells with opened stomata is attributable to the high K^+ concentration, and the effect of ABA in stomatal closure was by causing a transient efflux of K^+ through the plasmalemma of guard cells. Since stomatal opening is inhibited by ABA through K^+ efflux, this effect can be alleviated by floating epidermal strips on a high K^+ solution (Murthy *et al.*, 1984).

Induction of Freezing Resistance by ABA in Cultured Plant Cells

We have seen in the preceding section how a phytohormone, ABA, exerts its effect on guard cells. The rapid stomatal response depends primarily on the movement of ions and the mechanism is not likely to involve any changes in protein synthesis since the observable effects on stomatal closure occur within a few minutes.

In contrast to the rapid stomatal response, the induction of freezing resistance by ABA has been shown to require several days before cells acquire the adaptation (Chen and Gusta, 1983). Cell suspension cultures of winter wheat, winter rye and bromegrass were able to survive temperatures as low as -30°C when treated with an optimum ABA concentration (75 µM) for 4 days at 20°C. A longer treatment in ABA did not increase cold hardiness. In contrast, cell suspension cultures which were cold hardened by exposure to low temperature (2°C) as opposed to ABA-treatment at 20°C could only tolerate temperatures lowered to -10°C.

This observation is a further indication that ABA may have a role in the adaptation of plants to physiological stress. Although the mechanism for the adaptation is not known, the slowness of this response

may indicate that it is likely to involve regulation of gene expression. Chen and Gusta (1983) showed that of ten species tested, only those species with the capability to cold harden to low temperatures would show a dramatic increase in cold hardiness after addition of ABA to suspension cultures. They suggested that low temperature or ABA could trigger a response in cells to develop the capability to cold harden if they possess the genetic system to respond. This is consistent with the observation that warm season species such as soybean which were not capable of hardening upon exposure to low temperature, were unable to survive freezing temperatures even after ABA treatment.

At the whole plant level, there is an obvious correlation between the increase in endogenous ABA concentrations and exposure of plants to low temperature stress, for example, in tomato plants (Daie and Campbell, 1981). This correlation supports the idea that ABA may influence on gene expression enabling the plant to protect itself against stressful freezing temperatures.

Acceleration of Adaptation to Salt by ABA in Tobacco Cells

Another slow response to ABA is the adaptation of cultured tobacco cells to growth in NaCl (Larosa *et al*., 1985). In 0.17M NaCl, cell suspension cultures of tobacco attained double the fresh weight in a shorter time if ABA (100 μM) was included in the medium. Furthermore, growth of cells which had been adapted to growing in a certain concentration of NaCl was not stimulated by ABA if placed in the same concentration of NaCl. The stimulation of growth by ABA was only observable if the adapted cells were transferred to medium containing a higher NaCl concentration than the one it had become adapted to.

The observed effect of ABA on the acceleration of adaptation of tobacco cells to salt is somewhat similar to the induction of freezing resistance in cells (Chen and Gusta, 1983) mentioned above. In both cases, ABA accelerated the adaptation process. Cold hardening is usually a slow process and may take several weeks of exposure to low temperature, whereas ABA not only shortened the length of the process to about 4 days, but also achieved a greater level of hardening.

Currently, there is no information available in the literature on specifically cold-induced proteins in plant cells. However, unique proteins have been shown to accumulate in plant cells adapted to NaCl stress, and these include a major $M_r \sim$ 26,000 protein which appeared to be stimulated by ABA (Ericson and Alfinito, 1984).

Singh *et al.* (1985) found that the $M_r \sim$ 26,000 polypeptide was synthesised in tobacco cells not only in response to NaCl stress, but also to water stress imposed by PEG treatment. Since the appearance of this polypeptide corresponded with the commencement of culture growth, it was suggested that the $M_r \sim$ 26,000 polypeptide was not a shock protein associated with physiological trauma (as in heat shock or anaerobic shock), but rather, a protein that is associated with physiological adaptation. This suggestion is consistent with the observation that salt-adapted tobacco cells continued synthesising the $M_r \sim$ 26,000 polypeptide for some time after inoculation into a NaCl-free medium (Ericson and Alfinito, 1985).

Responses to Phytohormones and Environmental Factors

It is well-established that ABA plays a role in diverse physiological responses throughout the life of plants, from dormancy right through to senescence and abscission (Addicott and Van Steveninck, 1983). Many kinds of environmental stress stimulate the biosynthesis of ABA, and the hormonal effects of ABA in causing stomatal closure and other long-term adaptation processes (to environmental extremes e.g. freezing, salt) seem to operate via rather different mechanisms. Rapid stomatal closure has been shown to take effect through loss of turgor and extrusion of K^+ ions from guard cells, and it is reasonable to speculate that the process would not involve changes in gene expression. The accelerated adaptation of cells to salt stress in the presence of ABA was shown to be correlated with the synthesis of specific proteins and this may also be the case for induction of freezing resistance although no evidence is yet available.

Several plant systems have been studied to understand the mechanisms underlying changes in the spectrum of proteins synthesised in response to different environmental stresses. Simulated physiological conditions, such as variations in environmental extremes or changes in concentration of exogenous phytohormones may lead to cell responses manifested as an alteration in protein synthesis.

Translational Control of Alcohol Dehydrogenase by Anaerobiosis in Maize

Anaerobic treatment of maize seedlings causes selective synthesis of alcohol dehydrogenase (Sachs and Freeling, 1978; Sachs *et al.*, 1980). During the first hour of anaerobiosis, the synthesis of aerobic proteins was repressed accompanied by the appearance of new proteins (transition proteins) which became the major proteins synthesised. At 3-5 hours,

a further change in protein synthesis occurred: the appearance of a number of anaerobic proteins, the major ones being the alcohol dehydrogenase (ADH) enzymes. The change from the aerobic to the anaerobic pattern of protein synthesis gave a relatively simpler profile and remained the same until root death at about 70 hours.

In contrast to these *in vivo* results, *in vitro* translation of RNA from roots anaerobically treated for 5 hours stimulated the synthesis of both aerobic and anaerobic proteins. This indicated the availability of aerobic protein mRNA for translation although pulse-labelling experiments showed that aerobic protein synthesis was repressed during the first hour of anaerobiosis. Therefore, an early response in maize roots to anaerobic stress was to suppress translation of the "nonstress" protein mRNAs.

In maize roots, anaerobiosis served as a stimulus for the simultaneous induction of the anaerobic proteins and several of them have been identified including ADH1 and ADH2. The ADH1 gene product is a requirement for survival of maize plants in flooding (Schwartz, 1969), whereas the function of ADH2 is not known. In this system, regulation of protein synthesis occurred by changing both the translatability of particular mRNAs, as well as the levels of some of the mRNAs.

Regulation of Heat Shock Protein Synthesis in Soybeans

In soybean, a heat-shock response is shown by etiolated seedlings and in the leaves of mature plants (Kimpel and Key, 1985) when the temperature reaches a critical level. In

etiolated seedling hypocotyls, stepwise increases in temperature (28°C to 47.5°C, incrementing at 3°C hourly) cause heat shock (HS) proteins to be synthesised at 40°C. The profile of heat-shock proteins was similar to that of hypocotyls heat-shocked for 2 hours at 40°C (pulse-labeling data). A Northern analysis for seedlings exposed to stepwise increases in temperature also demonstrated a parallel increase with temperature in the steady state level of HS mRNAs, measured by hybridisation to cDNA clones for mRNAs which increased in abundance during a 2 hour heat-shock (40°C) regime.

The functions of these heat-shock proteins are unknown. In mature soybean plants, the temperature of induction for HS proteins is 5°C higher than that of seedlings. This is probably due to transpirational cooling which kept the tissue temperature lower than ambient. This idea is further supported by the observation on the differential response, over a 24 hour period, to temperature increases in the field by irrigated and non-irrigated plants. The leaves of non-irrigated plants contained higher levels of several HS mRNAs compared to irrigated plants.

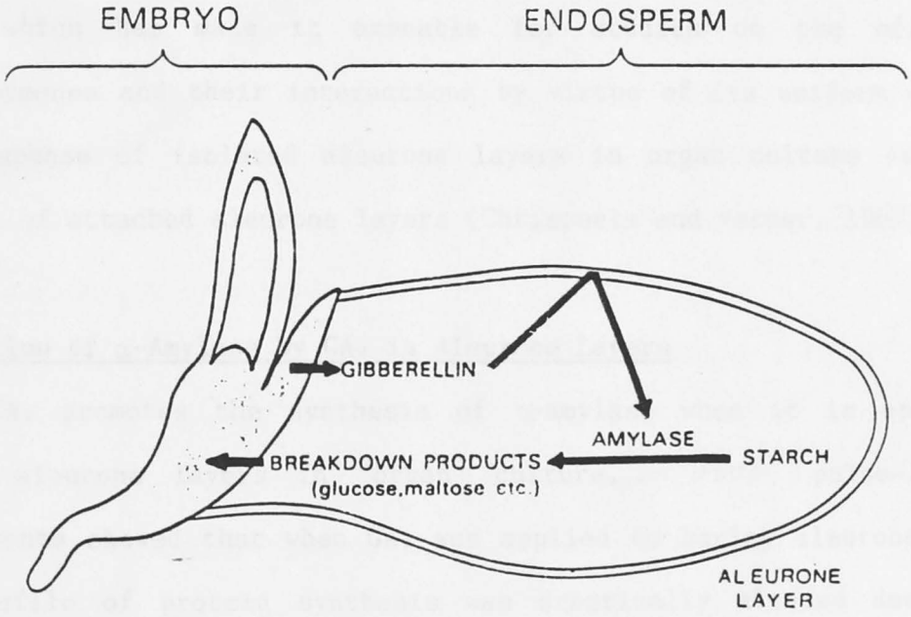
The accumulation of HS mRNAs in non-irrigated plants was transient. The steady state levels of the different species of HS mRNAs were highest at the hottest time of the day when ambient temperature reached 40°C. As the air temperature dropped, the HS mRNA levels dropped significantly. It is possible to envisage that the HS mRNA levels were a response to water stress. This is a complex situation where different environmental stresses could interact to affect plant-water relations. Nevertheless, Kimpel and Key (1985) were able to show that water stress on its own did not induce HS mRNAs which showed specific hybridization to probes representing HS mRNA sequences of soybean.

The observation that HS mRNAs were induced in field-grown plants suggests that HS proteins are produced as a normal plant response to high temperature.

Relationship Between Cell Responses to Heat Shock, Injury, Water Stress and ABA in Maize

In a different system, namely maize seedlings, novel proteins are induced by heat shock, injury (cutting), water stress and ABA (Heikkila *et al.*, 1984). In maize mesocotyls, heat shock stimulated the production of several specific heat shock proteins and also the enhancement of two proteins of $M_r \sim 70,000$. These two proteins were also induced by water stress and ABA although the imposition of either treatment also caused the stimulation of several other proteins unique to water stress and ABA treatment. The mRNA analysis for HS proteins was done by hybridization of mRNA isolated from mesocotyls of maize seedlings to a probe prepared from a cloned genomic DNA of the 5' end of the $M_r \sim 70,000$ *Drosophila* HS protein gene. The Northern analysis showed that mRNAs isolated from seedlings subjected to heat shock, water stress, ABA and injury (by cutting) accumulated mRNAs homologous to the HS protein gene in *Drosophila*. ABA was used as one of the stress-producing regimes because of the effect of water stress on ABA levels (see section above) and the implication this might have on the role of ABA in stress adaptation in plants. Heikkila *et al*. (1984) suggested that the accumulation of mRNA for the stress proteins in response to diverse stresses is regulated at the transcriptional level.

Germination of Barley Grains



When a barley grain imbibes water, the embryo releases a phytohormone, called gibberellin which stimulates the aleurone layer to synthesise and secrete several hydrolytic enzymes such as α-amylase, proteases and β-glucanase (Jacobsen, 1983; Fincher *et al.* 1986). The stored reserves in the starchy endosperm are broken down to sugars and amino acids to nourish the growing seedling.

The Aleurone Layer as an Experimental System

The aleurone layer surrounding the starchy endosperm can be isolated from the rest of the grain after hydration. Aleurone layers are isolated from de-embryonated half-grains so that the effects of exogenously supplied phytohormones can be studied without interference from phytohormones released by the embryo. The aleurone layer is comprised of about three layers of non-dividing similarly-differentiated cells which has made it amenable for studies on the effects of phytohormones and their interactions by virtue of its uniform response. The response of isolated aleurone layers in organ culture is similar to that of attached aleurone layers (Chrispeels and Varner, 1967).

Regulation of α-Amylase by GA_3 in Aleurone Layers

GA_3 promotes the synthesis of α-amylase when it is applied to barley aleurone layers in organ culture. *In vivo* pulse-labelling experiments showed that when GA_3 was applied to barley aleurone layers, the profile of protein synthesis was drastically altered due to the decreased and increased rates of synthesis of various proteins (Higgins *et al.*, 1982). The most striking GA-promoted protein was α-amylase, which made up 60% of total protein synthesised. The fact that maximal stimulation of α-amylase synthesis was not evident until about 12 hours suggested that the regulation of α-amylase synthesis involved changes in gene expression.

One avenue of investigation was to look at the level of translatable α-amylase message in a wheat germ translation system. It was found that the maximum level of translatable α-amylase mRNA constituted approximately 20% of the total translatable mRNA (Higgins

et al., 1982). This maximum level of translatable α-amlase mRNA level was attained at 6 h after GA_3 application. The significance of the large discrepancy between α-amylase mRNA levels (translatable only) and α-amylase levels is unknown. It is probable that in the aleurone layer geared to supply α-amylase for starch hydrolysis at the commencement of seedling growth, the translation of α-amylase translation may be favoured. This may operate through the presence of certain translation initiation sequences that facilitate greater efficiency of translation. Also, GA_3 may have other effects, such as stimulating the production of initiation factors that could increase the efficiency of α-amylase mRNA translation.

With the advent of recombinant DNA technology, total α-amylase message can be measured directly by hybridisation with a radioactive cDNA probe. Hybridization of size-fractionated RNA isolated from control and GA_3-incubated aleurone to an α-amylase cDNA clone that hybridized mostly to high pI (isoelectric point) α-amylase mRNAs revealed that the size of the α-amylase messages is approximately 1500 nucleotides and that they increased about 50-fold in abundance over control levels (Chandler *et al.*, 1984). This evidence supports results of the *in vitro* translation studies, that GA_3 modulates α-amylase accumulation primarily by influencing the level of α-amylase mRNA. However, it was not possible to determine whether the increase in α-amylase mRNA was due to a change in the rate of message transcription, processing of transcripts or degradation.

The genes for the different α-amylase isozyme groups are located on different barley chromosomes. Genes for the low pI isozymes of

α-amylase are located on chromosome 1, whilst genes for the high pI isozymes are located on chromosome 6 (Brown and Jacobsen, 1982; Muthukrishnan *et al.*, 1984). mRNA analysis by hybridization of aleurone RNA with radioactive high pI- or low pI- specific α-amylase cDNA clones showed that the mRNAs for the two isozymes accumulated at different rates and responded differently to varying concentrations of GA_3 (Huang *et al.*, 1984).

Regulation of α-Amylase by GA_3 in Aleurone Protoplasts

GA-responsiveness is also demonstrated by the aleurone protoplasts of oat (Hooley, 1982) and barley (Jacobsen *et al.*, 1985). In both systems, the characteristics of the response of crude protoplast preparations were similar to those of intact aleurone cells.

Barley aleurone protoplasts respond to GA_3 in the same manner as barley aleurone layers, by producing a new profile of protein synthesis, with α-amylase being the major pulse-labelled protein both within the protoplasts as well as secreted into the incubation medium (Jacobsen *et al.*, 1985). For unknown reasons, control protoplasts incubated under nitrogen synthesised a lower level of α-amylase than those in air. However, the N_2-protoplasts, in response to GA_3, achieved an α-amylase synthesis rate similar to that of air-protoplasts, so that the desired difference between control and GA_3-protoplasts was enhanced. Thus, barley aleurone protoplasts offer a good system for the study of phytohormones since in this system, too, changes in protein synthesis are induced by GA_3. The enhanced α-amylase production is accompanied by accumulation of its mRNA, which was shown to co-migrate with authentic α-amylase mRNA.

Zwar and Hooley (1986) showed that when nuclei were prepared from 72 h GA_4-treated oat aleurone protoplasts, the ^{32}P-labelled run-off transcripts contained 90 ± 10 ppm which hybridized to phage DNA containing α-amylase cDNA insert. In control nuclei, there was no significant hybridization of any RNA species to the α-amylase cDNA. This indicated that either no α-amylase mRNA was being transcribed or that α-amylase transcription was occuring below the detectable level of the system used, and that GA_4 promoted the transcription of α-amylase.

The advances made in oat protoplasts for transcription studies are also applicable to barley protoplasts (Jacobsen and Beach, 1985). α-amylase specific transcripts were detectable by hybridization to an α-amylase cDNA and were synthesised in much greater abundance in nuclei isolated from GA_3-treated protoplasts, compared to control. Furthermore, it was shown that the GA_3-increased α-amylase transcription was not due to an overall transcriptional increase, since the transcription of ribosomal RNA genes decreased in GA_3-treated protoplasts. These observations provided evidence that GA_3 regulates the transcription of both α-amylase and rRNA genes. The reduced total RNA transcription may be due to lowered rRNA transcription. This lends support to the observation that GA_3 has multiple effects in the transcription of genes in aleurones - suppressing some, and enhancing others.

In the aleurone system described above, various lines of evidence were used to show the fairly close correlation between levels of synthesis of particular proteins and their corresponding mRNAs. This correlation suggests that protein synthesis is regulated primarily at the level of transcription, with subsequent effects on mRNA abundance.

The Effect of ABA on Protein Synthesis in the Aleurone System

ABA induces the synthesis of several proteins and suppresses the synthesis of GA_3-promoted α-amylase in barley aleurone layers (Higgins *et al.*, 1982). When ABA is present in a 25-fold molar excess, GA_3-promoted changes in protein synthesis were reversed to resemble that of the control profile. The requirement for a 25-fold molar excess of ABA over GA may be due to several factors (1) the two hormones may be physiologically active at different concentrations, (2) the rate of uptake by cells may be different or, (3) the two hormones may be metabolised at different rates.

The change in polypeptide profile observed when aleurone layers were incubated in ABA was shown to be due to changes in the translatable mRNA populations, as demonstrated by *in vitro* translation studies (Higgins *et al.*, 1982). One of the ABA-induced polypeptides has been identified, i.e. the bifunctional α-amylase/subtilisin inhibitor (ASI), (Mundy, 1984). The mRNA level of ASI has been shown to be stimulated by ABA, but suppressed by GA_3 (Mundy *et al.*, 1986). The amino acid sequence of ASI has been determined by Svendsen *et al.* (1986) who also suggested that the α-amylase inhibitor and protease inhibitor sites may be sufficiently far apart so that the two functions do not interfere with each other. The locations of either of these active (or inhibitory) sites in ASI are unknown although by analogy with the soybean trypsin inhibitor, the protease inhibition site in ASI could be located between Val_{67} and Ala_{68}.

Identification of ASI polypeptide that had been translated in a cell-free system indicated that its mRNA was present in isolated

aleurone layer from mature grain and that the ASI mRNA level was increased by ABA (Mundy *et al.*, 1986). This is an indication that ASI may have a function in the prevention of precocious germination, through its inhibition of α-amylase activity. This suggestion is supported by the correlation between low endogenous ABA levels and precocious germination in viviparous mutants of maize (Wilson *et al.*, 1973).

Two other ABA-induced proteins have been identified. One of these, wheat germ agglutinin (WGA), is induced by ABA in cultured wheat embryos (Raikhel *et al.*, 1986). In this system, the introduction of a herbicide, fluoridone which inhibits carotenoid biosynthesis, caused WGA levels to be lower than in controls. Since ABA biosynthesis may occur via the breakdown of carotenoids, this provided circumstantial evidence for the involvement of endogenous ABA in regulating WGA levels in wheat embryo.

Another ABA-induced protein found in wheat embryo is the 'early-methionine-labelled' (E_m) polypeptide (Williamson *et al.*, 1985). The level of polypeptide synthesised is modulated primarily at the level of E_m mRNA abundance due to increased transcription or message stability.

Thus, the regulation of synthesis of several characterized and uncharacterized proteins has been shown to be regulated by the two plant growth regulators, ABA and GA_3, and the evidence available so far, indicates that the control of gene expression operates primarily at the level of mRNA abundance.

AIM OF STUDY

In this project, cultured aleurone layers from mature rehydrated barley grains are used as an experimental system for reasons mentioned in the Introduction. Barley aleurone layers respond to GA_3 and ABA in organ culture in the same way as if still attached to the grain, thus allowing simulation of events occurring during germination.

The aleurone system has been adopted in the study of α-amylase gene regulation by gibberellin, and this area of research has progressed to the extent that studies are currently being done to determine the regulatory segments of the α-amylase gene which interact with GA_3.

In contrast to the knowledge acquired on the effects of GA_3 on gene expression, very little is known about the effects and role of ABA. It is now known that GA_3 and ABA are antagonists: GA_3 promotes germination, while the presence of excess ABA in a grain negates the effect of GA_3 (i.e. germination is inhibited). ABA is particularly relevant in germination and plant water relations as demonstrated in the viviparous mutants of maize (Wilson *et al.*, 1973) and the *flacca* mutants of tomato (Tal and Imber, 1971; Imber and Tal, 1971).

The induction of α-amylase synthesis in barley aleurone layers by GA_3 is controlled primarily at the transcriptional level (Higgins *et al.*, 1982; Chandler *et al.*, 1984; Jacobsen and Beach, 1985). This project is designed to investigate, in greater detail, the effect of ABA on protein synthesis in parallel to previous work on GA_3 in barley aleurone layers. cDNA clones for ABA-induced messages have been constructed by Dr P.M. Chandler, and these clones were characterized prior to their use as probes in hybridization analyses to study gene

regulation at the RNA level. In addition, one of these clones was selected for sequencing and the deduced amino acid sequence was compared with the known amino acid sequences of other proteins in the Data Bank, so that the function of the protein coded for by the cDNA might be elucidated. Also, since metabolism of ABA affects the ABA concentration (and therefore, its activity) through conversion to PA and DPA, the effect of PA on protein synthesis was also studied. The long-term aim of these studies is to make some progress in the understanding of the mode of action of ABA.

This thesis is organised as follows: Chapter 1 focuses on changes in protein synthesis promoted by ABA and PA in the aleurone layer as seen by *in vivo* pulse-labelling with [^{35}S]methionine. In Chapter 2, the changes in protein synthesis are investigated at the RNA level, by *in vitro* translation studies, and hybridisation studies. Finally, in Further Discussion, issues relevant to the results obtained are addressed in the context of current knowledge on hormone action.

CHAPTER 1

INTRODUCTION

In barley aleurone layers, ABA induces the synthesis of several polypeptides and suppresses the synthesis of others, notably that of the GA_3-promoted polypeptide, α-amylase (Higgins *et al.*, 1982; Mundy, 1984). Mundy (1984) estimated the ABA-induced polypeptides to have M_r's of ∿14,000, 20,000, 25,000, 28,000, 31,000, 35,000 and 50,000. One of the ABA-induced polypeptides, M_r ∿ 20,000 was identified as ASI (α-amylase/subtilisin inhibitor) which inhibits both the high pI family of barley α-amylase and the bacterial protease subtilisin. *In vivo* pulse-labelling studies by Higgins *et al.*, (1982) demonstrated the secretion of α-amylase by barley aleurone layers treated with GA_3, however, none of the ABA-induced polypeptides was secreted.

It is not known whether ABA directly induces the synthesis of polypeptides in barley aleurone layers or whether the active component is a metabolite of ABA. Uknes and Ho (1984) postulated that phaseic acid (PA), the first stable metabolite of ABA, is the active component that suppresses the expression of the α-amylase gene and they suggested the following scheme for ABA and PA action : ABA induces the synthesis of several proteins, one of which is an oxygenase which converts ABA to PA, which then inhibits the synthesis of α-amylase. Ho *et al.* (1985) subsequently showed that PA is inactive in inducing the synthesis of 'ABA polypeptides'.

In this chapter, the induction of protein synthesis by ABA was studied in detail by *in vivo* labelling techniques. Detailed studies on the effects of ABA included time-course for induction, dose response,

ABA-GA_3 interaction, heat-treatment of the ABA polypeptides and secretion. Studies on PA were done to compare its activity with that of ABA in barley aleurone layers, specifically, whether PA induced the synthesis of the 'ABA polypeptides'.

MATERIALS AND METHODS

Isolation of Aleurone Layers

Barley grains (*Hordeum vulgare* cv. Himalaya, 1984 harvest obtained from Dr J.V. Jacobsen, CSIRO Division of Plant Industry, Canberra) were cut at both the apex of the distal half as well as tranversely to remove the embryo. De-embryonated half-grains were surface sterilised for 30 minutes with 1% sodium hypochlorite containing a few drops of TWEEN 20, rinsed with six washes of sterile distilled water, soaked in filter-sterilised 10 mM HCl for 10 minutes, followed by six rinses in sterile distilled water. Half-grains were hydrated on sterile filter paper in a petri dish containing 5 ml of sterile distilled water, at room temperature for 4 days.

The aleurone layers were removed from the endosperm using two spatulas as described (Chrispeels and Varner, 1967). Aleurone layers (5 or 10) were incubated in 2 ml of the appropriate medium in a 25 ml Erlenmeyer flask at 25°C with shaking at 70-75 oscillations per minute. Incubation media contained 10 mM $CaCl_2$, 10 µg/ml chloramphenicol with or without the addition of ABA ((±) cis-trans isomer >99%, Sigma Chemical Co.), GA_3 (Sigma Chemical Co.) or PA (obtained from two sources: B.R. Loveys of the CSIRO Division of Horticultural Research, Adelaide, South Australia and J.A.D. Zeevaart, MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan, USA).

In vivo Labelling and Protein Extraction

Aleurone layers were prepared and incubated as above. Prior to labelling, the old incubation medium was removed from each flask by aspiration, and aleurone layers washed with 3 x 5 ml distilled water. Flasks were then incubated for the chosen duration of 2, 4 or 6 h labelling in 1 ml of fresh incubation medium and 1.1 MBq of [^{35}S]-methionine. The aleurone layers were finally washed with 5 ml of 10 mM $CaCl_2$, and ground in a mortar and pestle in 1.5 ml buffer containing 20 mM N-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES)-KOH (pH 8.0), 0.5 M NaCl and 1 mM phenylmethyl-sulfonyl fluoride (PMSF). Homogenates were transferred to 1.5 ml microfuge tubes and kept on ice with occasional agitation for 30 minutes. After a 10 minute centrifugation (12,000 x g), aliquots of the supernatant (total protein extracts) were assayed for protein (Lowry *et al.*, 1951) and trichloroacetic acid (TCA)-precipitable counts (Mans and Novelli, 1961).

Preparation of Protein Extracts for SDS-Polyacrylamide Gel Electrophoresis

For heat treatment of protein extracts, a fraction of the supernatant was heated at 70°C for 10 minutes, left on ice for 30 min., and centrifuged (12,000 x g) for 5 min. Supernatants of heated and nonheated samples were precipitated with 4 volumes of acetone at -20°C overnight, centrifuged at 12,000 x g for 10 min. and pellets washed with 5% cold TCA, then with 80% acetone containing 2 mM methionine and dried in a vacuum desiccator.

Preparation of Secreted Proteins for SDS-Polyacrylamide Gel Electrophoresis

At the end of the labelling period, and following removal of aleurone layers, the incubation medium was centrifuged for 5 min. in a microfuge to remove aleurone layer 'debris'. Protein in the supernatant was precipitated with 4 volumes of acetone at -20°C overnight and prepared for electrophoresis as described for total protein extracts. Samples of media protein were not heat-treated.

SDS-Polyacrylamide Gel Electrophoresis

Pellets of the precipitated protein extracts were dissolved in dye-SDS buffer (125 mM Tris-HCl pH 6.7, 2% SDS, 10% glycerol, 0.01% w/v bromphenol blue, 5% mercaptoethanol) and heated at 80°C for 3 min. SDS-polyacrylamide gel electrophoresis was carried out according to the procedure of Spencer *et al.* (1980), with modifications. The slab gradient polycrylamide gels (12.5% to 25% w/v) were of dimensions 150 x 230 x 1 mm. The final concentration of Tris (Ultrol, Calbiochem) was 0.39 µM in the separating gel. The stock acrylamide solution which contained 50% acrylamide and 0.66% N,N'-Methylene bis-acrylamide (Eastman Kodak Co., Rochester, USA) was treated with charcoal (Norit A, acid washed) and filtered through Whatman filter paper (No. 50). The heavy solution (total volume 17.7 ml) was made up from 8.8 ml stock acrylamide (as specified above), 4.6 ml of 1.5 M Tris-HCl pH 8.4 at 20°C, 184 µl of 10% SDS (Specially Pure, BDH Chemicals Australia Pty. Ltd.), 5 g of glycerol, 16 µl of 10% ammonium persulphate and 16 µl of TEMED. The light solution contained 4.6 ml stock acrylamide, 4.6 ml of 1.5 M

Tris-HCl pH 8.4, 8.9 ml of water, 184 µl of 10% SDS, 24 µl of 10% ammomium persulphate and 20 µl of TEMED. The gradient gel was made up using a polystaltic pump, overlaid with 0.1% SDS and left to polymerise for 2 h. The stacking gel (6% acrylamide) was made up of 3.8 ml of acrylamide solution (25% acrylamide, 0.66% bisacrylamide), 4.0 ml of 0.5 M Tris-HCl pH 6.7 (at 20°C), 8.0 ml of water (the mixture was degassed at this stage), 160 µl of 10% SDS, 160 µl of 10% ammonium persulphate and 16 µl of TEMED. The stacking gel was allowed to polymerise for 1 hour. The reservoir buffer contained Tris (Trizma base Sigma 7-9) (3 g/1), glycine (14 g/1) and 0.05% SDS (final concentration). The gel was electrophoresed at 8 mA for the first hour, and at 12-17 mA for 16 hours, or until the bromphenol blue dye had run off the bottom of the gel.

The gel was washed twice (with shaking) in destainer (2 l contained 500 ml ethanol, 140 ml glacial acetic acid and 1360 ml water) then stained in a solution containing 0.5% Coomassie brilliant blue R in 10% acetic acid, 50% ethanol (v/v) for 45 minutes.

The gel was prepared for fluorography by a modification of the method of Bonner and Laskey (1974), dried on a gel drier for 1.5 h at 60° and exposed to Fuji RX X-ray film at -80° for the appropriate length of time (several days to several weeks).

RESULTS

Induction of Protein Synthesis by ABA

Figure 1.1a shows the pattern of protein synthesis in aleurone layers which were incubated for 16 h without hormone [C], with 25 μM ABA [+ABA] or with 1 μM GA_3 (+GA). Note that treatment of aleurone layers with ABA or GA_3 caused a dramatic change in the polypeptide profile compared with the [C] layers. ABA induced the synthesis of several polypeptides and suppressed the synthesis of others. Some of these ABA-induced polypeptides, to be collectively called 'ABA polypeptides', were apparently synthesised but at lower levels in [C] aleurone layers e.g. $M_r \sim 26,000$; others were not visible in the [C] tracks e.g. several polypeptides at $M_r \sim 75,000$.

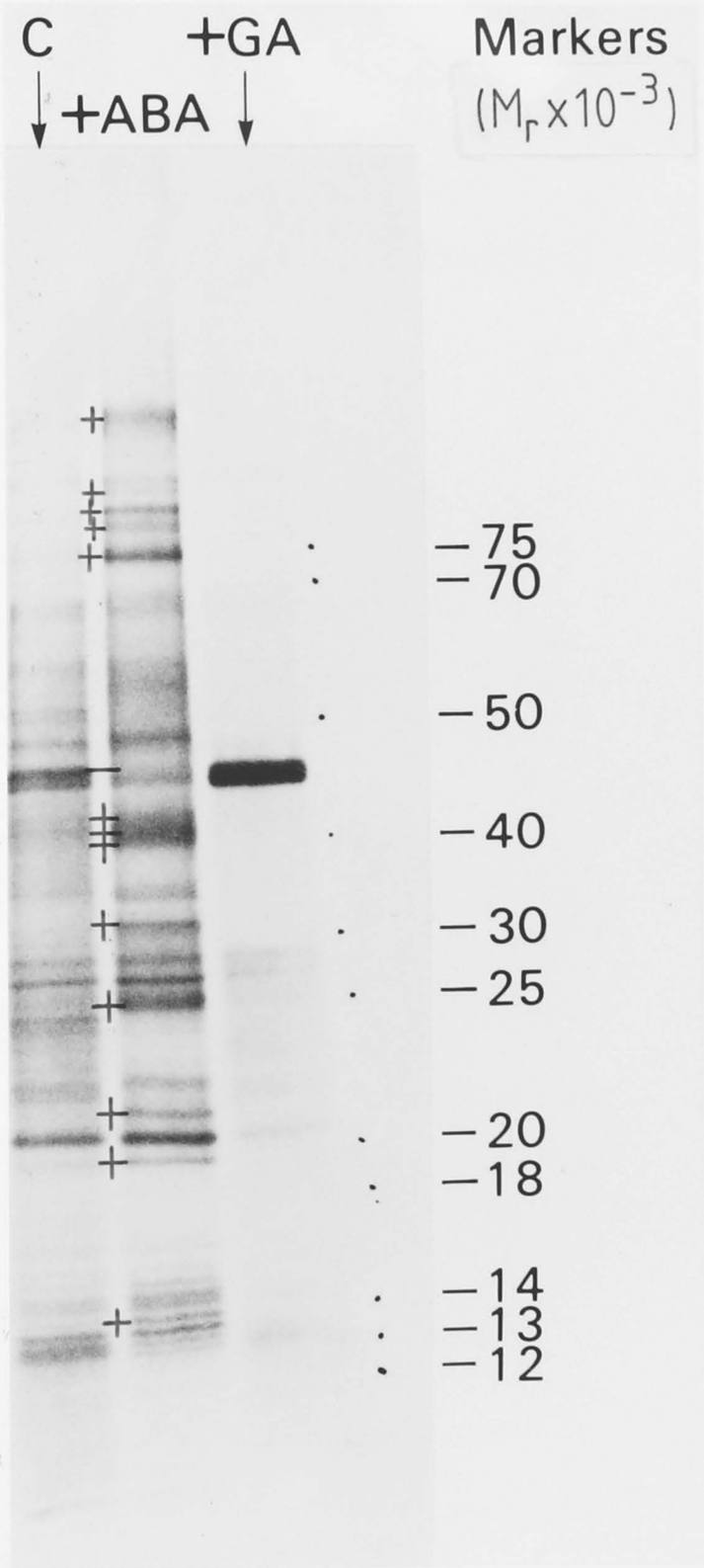
ABA also suppressed the synthesis of a polypeptides of $M_r \sim 44,000$. Synthesis of the $M_r \sim 44,000$ polypeptide was promoted by GA_3, and it corresponds to α-amylase since it has a similar abundance and electrophoretic mobility as the ABA-suppressed protein (at $M_r \sim 44,000$) identified as α-amylase (immunologically and biochemically) by Higgins *et al.* (1982).

J.V. Jacobsen (personal communication) has recently shown that most of the ABA-induced polypeptides remained soluble after heat treatment of total protein extracts, whereas a majority of other proteins coagulate. Heat treatment (see Figure 1.1c) of the [-ABA] protein extract resulted in the loss (by precipitation) of all polypeptides, including α-amylase at $M_r \sim 44,000$, seen in the non-heated [-ABA] track (cf. Figure 1.1b). However, although heat treatment of [+ABA] protein extract resulted in the loss of some ABA-induced polypeptides, the changes induced by ABA in the overall pattern of protein synthesis have become clearer.

Figure 1.1a Fluorogram of an SDS-polyacrylamide gel of the polypeptides (non-heated) from (Control), (+ABA) and (+GA) aleurone layers. Aleurone layers were incubated without hormone, with 25 μM ABA or 1 μM GA_3 and pulse-labelled with [^{35}S]methionine during the last 2 h of a 16 h incubation. Tracks were loaded for equal TCA-precipitable cpm. Numbers ($M_r \times 10^{-3}$) indicate positions of protein mobility markers.

+ indicates ABA-induced polypeptides.

- indicates ABA-suppressed polypeptides.


C
+GA
+ABA
Markers
($M_r \times 10^{-3}$)
75
70
50
40
30
25
20
18
14
13
12

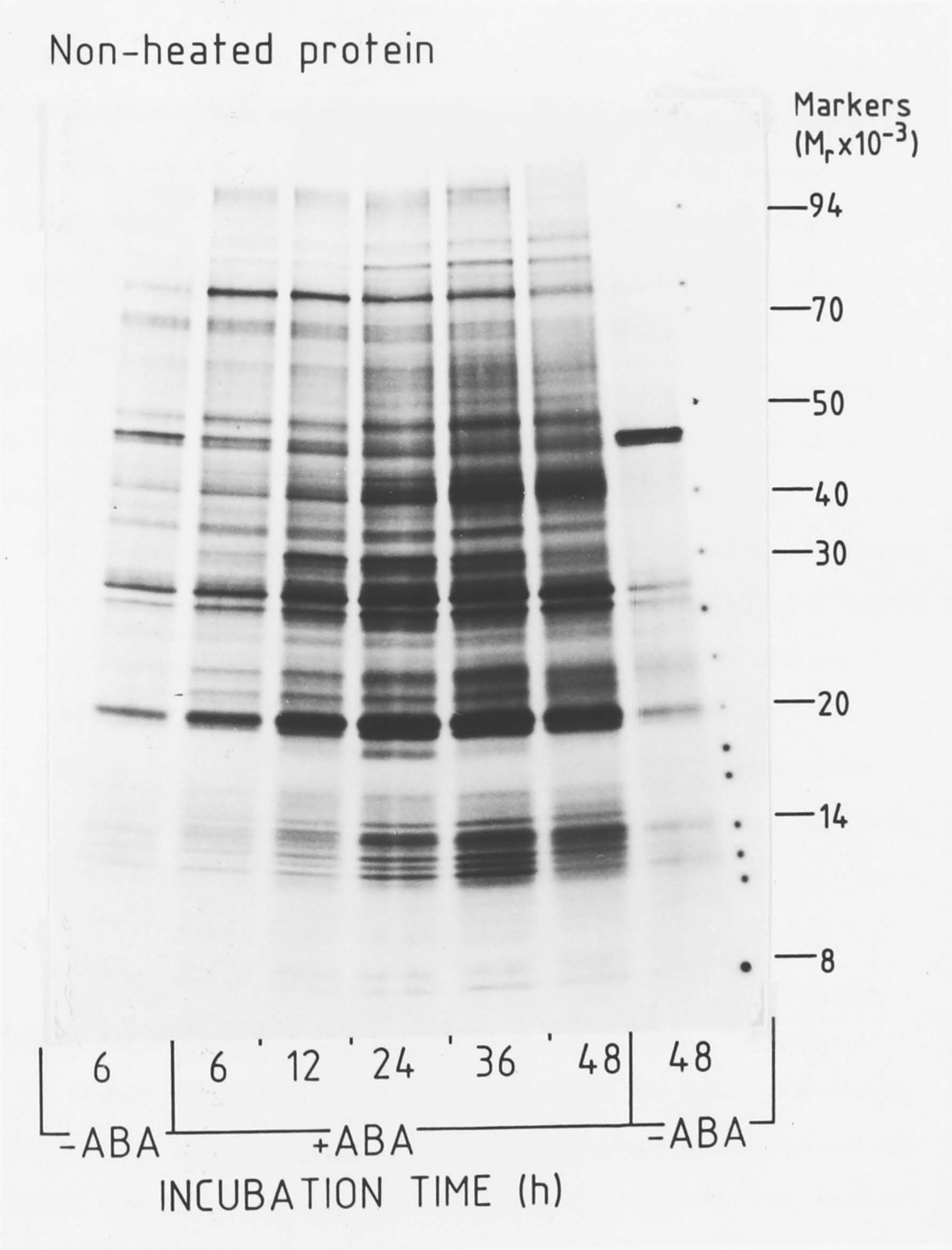
Tracks in Figs. 1.1b and 1.1c were loaded to represent 0.1 aleurone layer equivalents of pulse-labelled protein. The specific activity of *in vivo* labelled polypeptides may be affected by amino acid pools which may vary in size according to incubation conditions. Expansion of the amino acid pools occurs in GA_3-treated aleurone layers due to increased proteolysis. Fig. 1.1a shows tracks loaded for equal TCA-precipitable cpm for protein extracted from [Control], [+ABA] and [+GA] aleurone layers. There is a two-fold difference in the specific activity of protein extracted from [+ABA] aleurone layers compared with that of [Control] layers (unpublished data). Comparison of Figs. 1.1a and 1.1c showed that whether samples were loaded for equal aleurone equivalents or equal TCA-precipitable cpm (to compensate for any differences in specific activity of proteins synthesised), it is clear that ABA induced the synthesis of several polypeptides and suppressed the synthesis of α-amylase.

Time-Course of Appearance of 'ABA Polypeptides'

Changes in the polypeptide profile resulting from ABA treatment are likely to involve effects on gene expression, or perhaps changes in stability of mRNAs. To investigate the kinetics of these changes the pattern of protein synthesis was examined in aleurone layers incubated without ABA for 6 h and 48 h, and with 25 μM ABA for different incubation times.

There were notable differences (Fig. 1.2a) in the polypeptide profiles of the [-ABA] aleurone layers at 6 h and 48 h. The synthesis of low levels of ABA-induced polypeptides in the 6 h [-ABA] track may be due to residual ABA or ABA-induced mRNAs, accumulated during the

Figure 1.2(a) Fluorogram of an SDS-polyacrylmide gel showing a time-course of appearance of ABA-induced proteins. Aleurone layers were incubated without hormone or with 25 μM ABA for the indicated time and pulse-labelled with [^{35}S]methionine for the last 6 h of incubation. Each track was loaded to represent non-heated protein extracted from 0.1 aleurone layer equivalents. Numbers ($M_r \times 10^{-3}$) indicate positions of protein mobility markers.


Non-heated protein
Markers
(M_r x 10^{-3})
94
70
50
40
30
20
14
8
6
6
12
24
36
48
48
-ABA
+ABA
-ABA
INCUBATION TIME (h)

development of the grain, persisting at maturity (see Discussion, Chapter 1 and Results, Chapter 2). By 48 h, however, much of the "background" synthesis of ABA-induced polypeptides had declined and this may be explained by an expansion of amino acid pools since [-ABA] aleurone layers contain endogenous GA_3. There was an increase in the level of α-amylase synthesis in response to endogenous GA_3 present in the aleurone.

In the presence of 25 μM ABA, there was a gradual change in the pattern of protein synthesis with different incubation times (Fig. 1.2a). These complex changes in the pattern of protein synthesis were more clearly seen after heat treatment of the protein extracts, as shown in Fig. 1.2b (most, but not all ABA-induced proteins were retained in solution after heating). Some polypeptides were induced soon after ABA treatment (e.g. $M_r \sim 34,000$ at 6 h) whereas others appeared at later times (e.g. two polypeptides $M_r \sim 76,000$ and 83,000 at 24 h). Following induction, the synthesis of some of these polypeptides declined fairly early (e.g. $M_r \sim 34,000$ at 24 to 36 h) whereas the group of polypeptides at $M_r \sim 40,000$ exhibited continued synthesis for at least 48 h in ABA. These results indicated that ABA-induced proteins may appear after a short (6 h) or a longer (24 h) incubation in ABA, and that synthesis may begin to decline as soon as 24 h or may contine for at least 48 h.

Requirement for a Threshold Concentration of ABA

In order to further study the action of ABA, the lowest concentration of ABA sufficient to change the polypeptide profile was determined. The dose-response for ABA (Fig. 1.3) showed that 0.33 μM ABA was sufficient to induce the characteristic ABA polypeptide profile

Figure 1.2(b) Fluorogram of an SDS-polyacrylamide gel of heated samples from the ABA induction time-course. Aleurone layers were incubated without hormone or with 25 μM ABA for the indicated time and pulse-labelled with [^{35}S]methionine for the last 6 h of incubation. Each track was loaded to represent protein extracted from 0.1 aleurone layer equivalents, and samples were heated at 70°C for 10 min and prepared for electrophoresis as described in Methods. Numbers ($M_r \times 10^{-3}$) indicate positions of protein mobility markers.

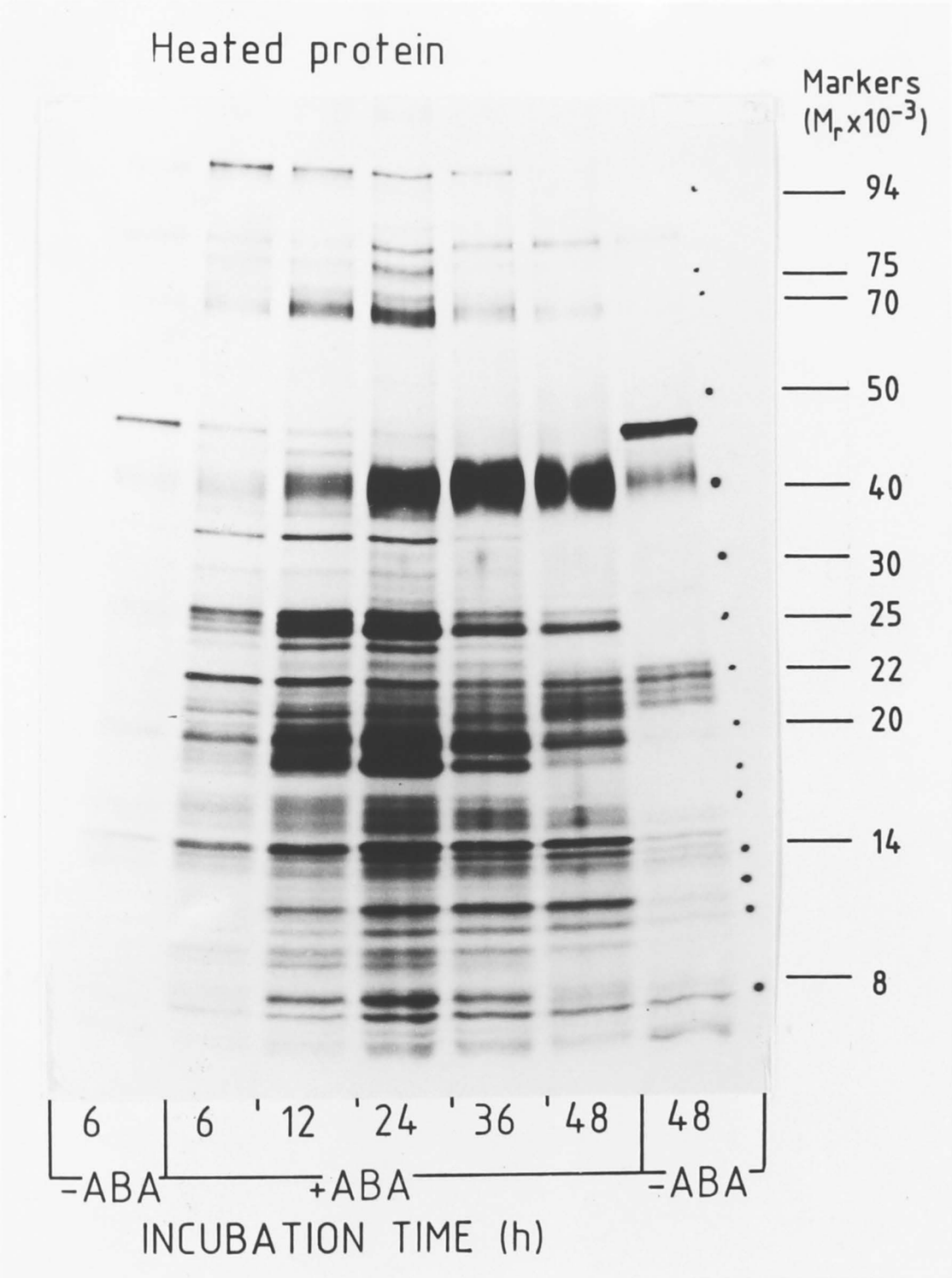
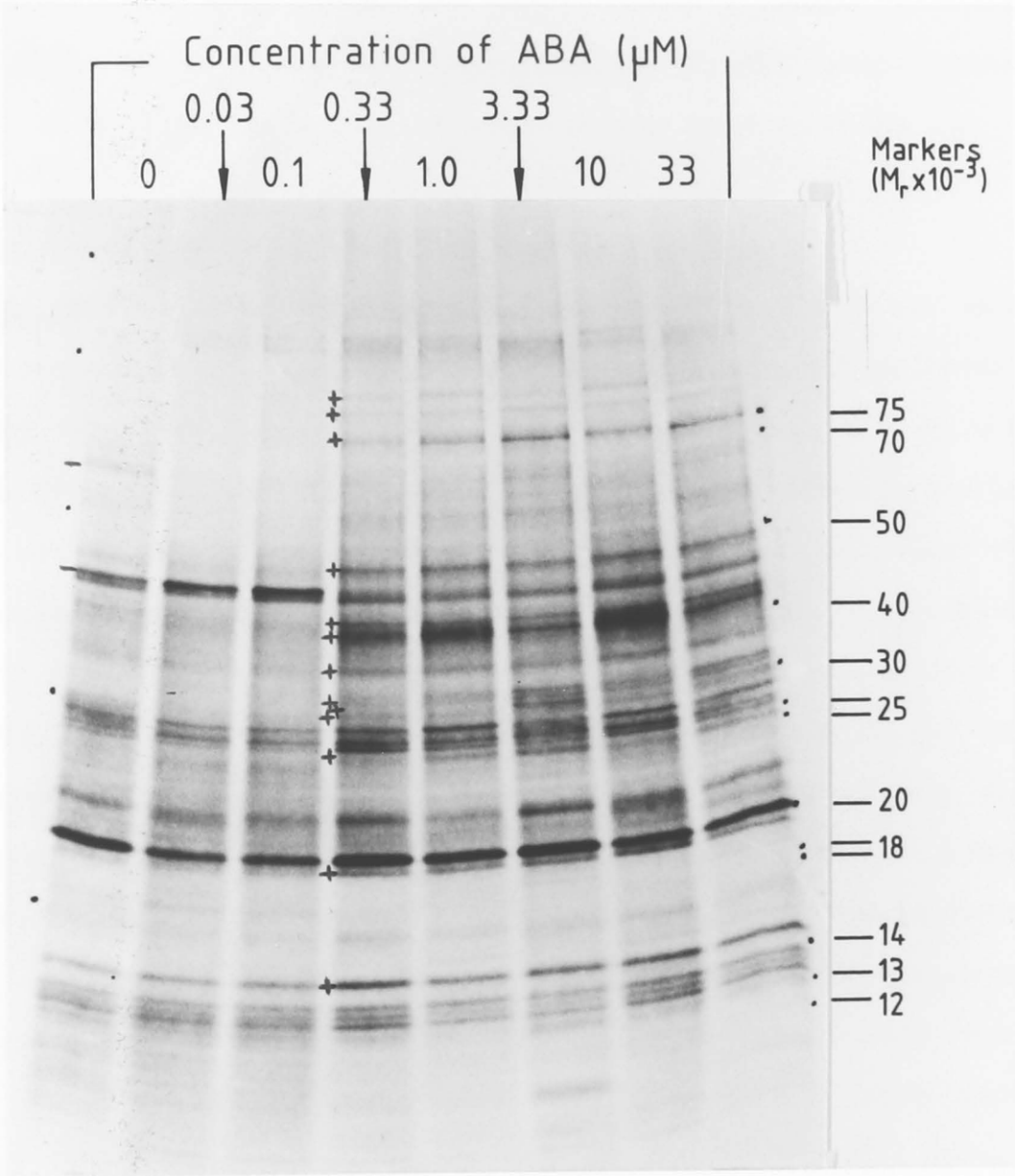

Heated protein
Markers
(M_rx10^{-3})
94
75
70
50
40
30
25
22
20
14
8
6
6
12
24
36
48
48
-ABA
+ABA
-ABA
INCUBATION TIME (h)

Figure 1.3 Fluorogram of an SDS-polyacrylamide gel of dosage response to ABA. Aleurone layers were incubated in 0 to 33 μM ABA and pulse-labelled with [^{35}S]methionine during the last 2 h of the 16 h incubation. Each track was loaded for equal TCA-precipitable counts. Numbers ($M_r \times 10^{-3}$) indicate positions of protein mobility markers.

\+ indicates ABA-induced polypeptides.

\- indicates ABA-suppressed polypeptides.


Concentration of ABA (μM)
0.03
0.33
3.33
0
0.1
1.0
10
33
Markers
(M_r x 10^{-3})
75
70
50
40
30
25
20
18
14
13
12

during a 16 h incubation. In addition, at 0.33 μM or higher concentrations of ABA, the upper band of the $M_r \sim 44,000$ doublet i.e. α-amylase, was no longer synthesised. This suggested that the synthesis of this particular polypeptide was suppressed when the ABA concentration (due to endogenous and exogenous ABA levels) reached a certain threshold value sufficient to induce the ABA polypeptides. At ABA concentrations of 0.33 μM to 33 μM, the polypeptide profiles were essentially the same.

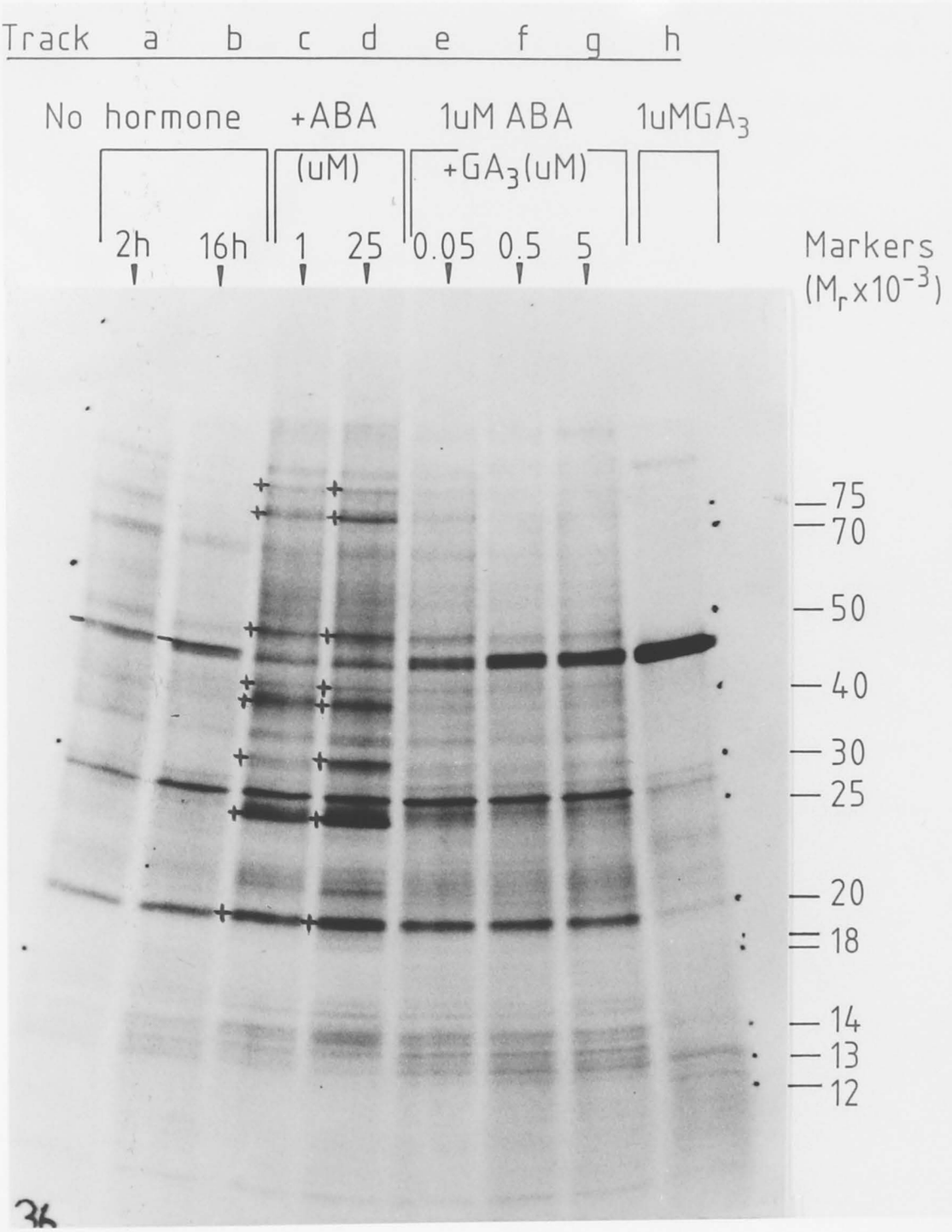
ABA-GA_3 Interaction

As the synthesis of several GA_3-induced proteins was not seen in the presence of ABA, the effect of GA_3 on the synthesis of ABA-induced proteins was investigated by incubating aleurone layers in 1 μM ABA with a range of concentrations of GA_3 (Fig. 1.4). As shown in tracks e, f and g, there was almost complete reversal of induction of polypeptides whose synthesis was stimulated by 1 μM ABA in the presence of 0.05 μM GA_3, and complete reversal at higher GA_3 concentrations. In this experiment some differences were seen in the degree of stimulation by 1 μM and 25 μM ABA (tracks c and d), however this was not consistently observed. Control tracks from incubations with no added hormone exhibited a low level of synthesis of ABA-induced polypeptides at 2 h (track a), and much less at 16 h (track b). As mentioned previously, this difference could be due to a number of factors. There was a concomitant rise in the level of α-amylase synthesis (upper band of $M_r \sim 44,000$ doublet) from 2 h to 16 h. In the presence of 1 μM GA_3 (track h), the level of α-amylase polypeptide increased many-fold in agreement with previous studies (Higgins *et al.*, 1982).

Figure 1.4 Fluorogram of an SDS-polyacrylamide gel of the interaction between ABA and GA_3. Aleurone layers were incubated for (a) 2 h without hormone or for 16 h in media containing, (b) no hormone, (c) 1 μM ABA, (d) 25 μM ABA, (e) 1 μM ABA + 0.05 μM GA_3, (f) 1 μM ABA + 0.5 μM GA_3, (g) 1 μM ABA + 5 μM GA_3, (h) 1 μM GA_3. Samples were pulse-labelled with [^{35}S]methionine during thelast 2 h of incubation. Each track was loaded for equal TCA-precipitable cpm. Numbers ($M_r \times 10^{-3}$) indicate positions of protein mobility markers.

\+ Indicates the polypeptides induced by 1 μM and 25 μM ABA.

\- Indicates the ABA-suppressed polypeptides at $M_r \sim 44{,}000$.


Track a b c d e f g h
No hormone
+ABA (uM)
1uM ABA +GA3(uM)
1uMGA3
2h
16h
1
25
0.05
0.5
5
Markers (Mr x10-3)
75
70
50
40
30
25
20
18
14
13
12

Effect of PA on Protein Synthesis

It has been suggested that PA, a metabolite of ABA, is the active component which reverses the GA_3-promoted synthesis of α-amylase, but that PA does not by itself induce the synthesis of new proteins (Ho *et al.*, 1985; Uknes and Ho, 1984).

Figure 1.5 shows the polypeptides of non-heated protein extracts from aleurone layers incubated for 16 h without additions, with 1 μM PA or ABA; or with 25 μM PA or ABA. In the control track, it can be seen that α-amylase was the most abundant polypeptide synthesised in aleurone layers during a 16 h incubation. The profile of polypeptides induced by PA was the same as that induced by ABA at the two concentrations used. This result indicated that PA also stimulated the synthesis of the 'ABA polypeptides' and the activity of PA was similar to that of ABA.

It has been shown (e.g. in Fig. 1.1) that most of the ABA-induced polypeptides remained in solution after heat treatment of total protein extracts. Figure 1.6 shows the polypeptide profiles of heated protein extracts from aleurone layers incubated for 48 h in 1 μM PA, 25 μM PA and 25 μM ABA. For heated protein extracts, the polypeptide profiles were the same for ABA- and PA-incubated aleurone layers. In the control track, most of the polypeptides precipitated after heat treatment, as expected. The results of Figure 1.5 and Figure 1.6 indicated that there was no difference between the polypeptide profiles induced by PA and ABA, whether the comparison was done with heated or non-heated protein extracts. Similar responses were obtained for aleurone layers incubated in two different preparations of PA which were obtained from B.R. Loveys

Figure 1.5 Fluorogram of an SDS-polyacrylamide gel of tissue proteins (non-heated samples) induced by ABA and PA. Aleurone layers were incubated without hormone (control), or with 1 μM ABA, 25 μM PA, or 25 μM ABA and pulse-labelled during the last 2 h of a 16 h incubation. Each track was loaded for equal TCA-precipitable cpm. Numbers ($M_r \times 10^{-3}$) indicate positions of protein mobility markers.

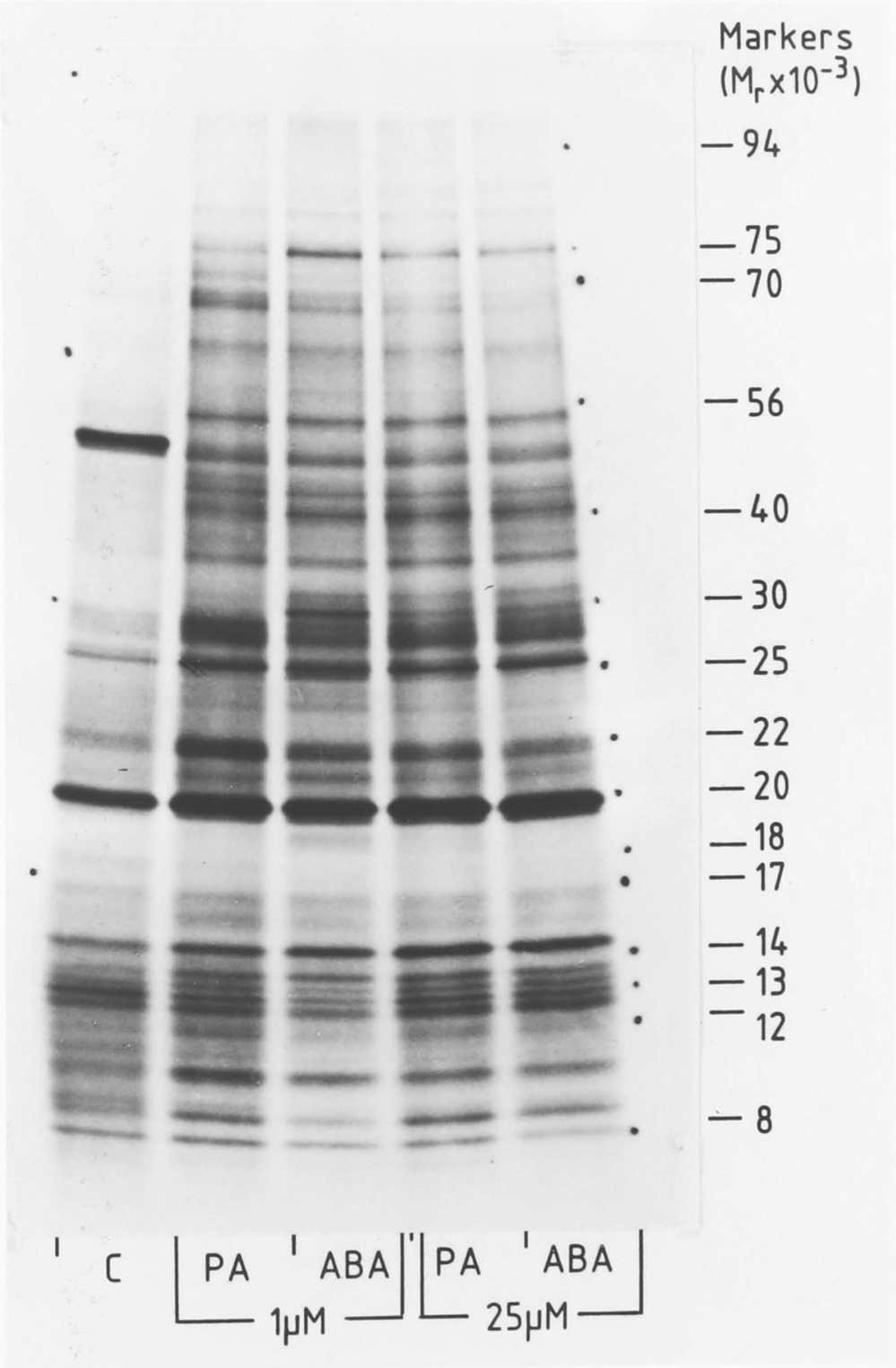
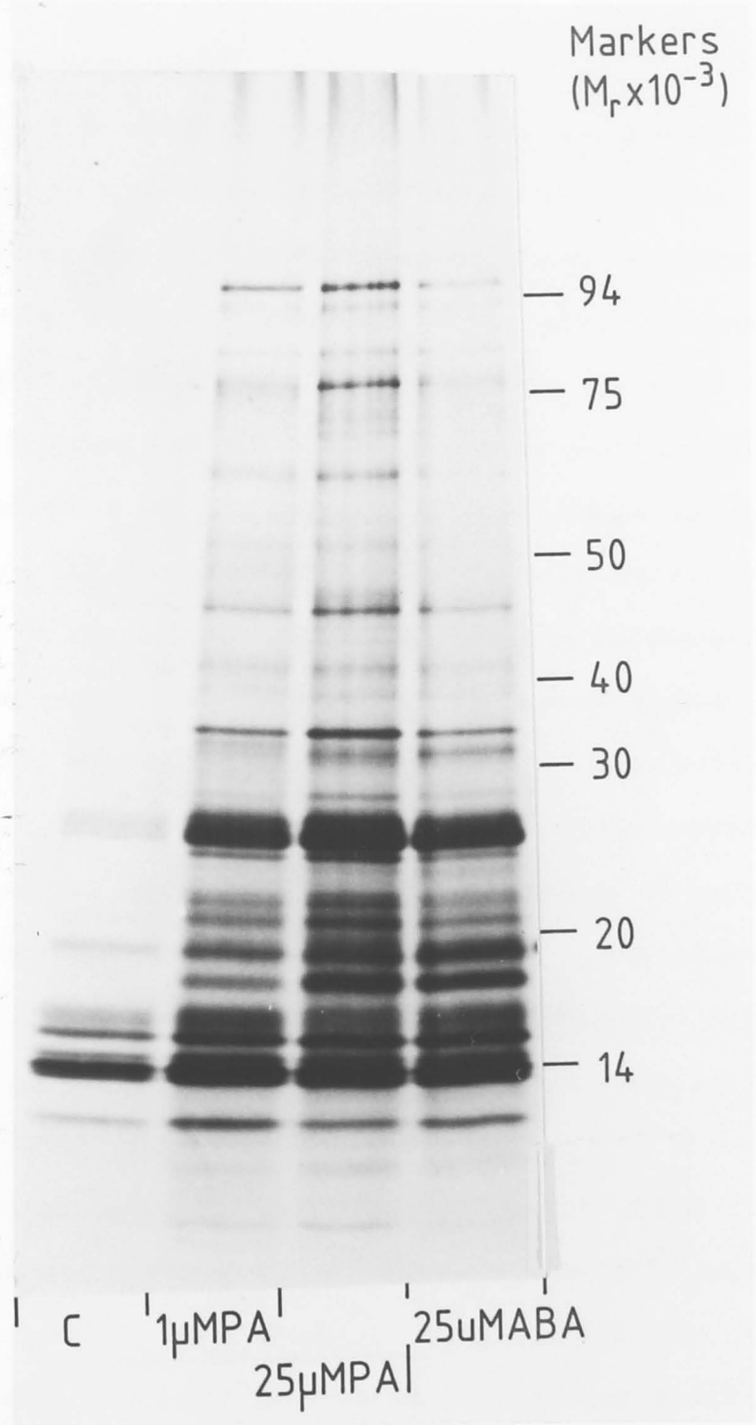

Markers
(M_r x 10^{-3})
94
75
70
56
40
30
25
22
20
18
17
14
13
12
8
C
PA
ABA
PA
ABA
1μM
25μM

Figure 1.6 Fluorogram of an SDS-polyacrylamide gel of tissue proteins induced by ABA and PA. Aleurone layers were incubated without hormone (control), 1 μM PA, 25 μM PA and 25 μM ABA and pulse-labelled with [^{35}S]methionine during the last 6 h of a 48 h incubation. Each track contains 0.1 aleurone layer equivalents of aleurone protein heated at 70°C for 10 min and prepared for electrophoresis as in Methods. Numbers ($M_r \times 10^{-3}$) indicate positions of protein mobility markers.


Markers
(M_rx10^{-3})
94
75
50
40
30
20
14
C
1μMPA
25μMPA
25uMABA

and J.A.D. Zeevaart. Therefore, in contrast to previous work (Ho *et al.*, 1985), PA was as effective as ABA in stimulating the synthesis of 'ABA polypeptides' in barley aleurone.

Secretion of 'ABA Polypeptides'

It is well-established that several of the GA_3-promoted enzymes, e.g. α-amylase and protease are secreted by aleurone layers. However, it has been suggested that ABA-induced proteins are not secreted (Higgins *et al.*, 1982). To further investigate this, aleurone layers were incubated for 48 h in 25 μM ABA, and protein was extracted from both aleurone layers (tissue) and the medium (secreted protein). Figure 1.7 showed the partitioning of polypeptides between tissue and media and demonstrates that a few of the total polypeptides were secreted.

Since some of the proteins have been shown to be secreted by the aleurone into the medium, the polypeptide profiles of protein secreted by [+PA] and [+ABA] aleurone layers were compared. Aleurone layers were incubated with ABA or PA for 48 h and secreted protein extracted from the incubation medium. Figure 1.8 shows the profiles of polypeptides secreted by aleurone layers incubated in ABA or PA. In the control track, α-amylase (M_r ∿ 45,000) was one of several polypeptides secreted by aleurone layers at 48 h of incubation. However, α-amylase was not secreted by aleurone layers treated with ABA or PA. There was no observable difference in the polypeptide profiles for secreted proteins of ABA- and PA-treated aleurone in which two new secreted polypeptides (M_r ∿ 40,000 and ∿30,000) were observed. These two ABA- and PA-induced secreted polypeptides were never observed in the secreted protein profile of control layers. However, the level of the M_r ∿ 20,000 polypeptide

Figure 1.7 Fluorogram of an SDS-polycrylamide gel of the comparison between tissue and secreted polypeptides for ABA-incubated layers. Aleurone layers were incubated with 25 μM ABA and pulse-labelled with [^{35}S]methionine during the last 6 h of the 48 h incubation. Protein was extracted from aleurone layers, heated at 70°C for 10 min and prepared for electrophoresis as described in Methods. Secreted protein was extracted from the incubation medium and was not heated. The tissue track was loaded to represent protein extracted from 0.1 aleurone layer equivalents and the medium track was loaded to represent protein extracted from 0.2 aleurone layer equivalents. Numbers (M_r x 10^{-3}) indicate positions for protein mobility markers.

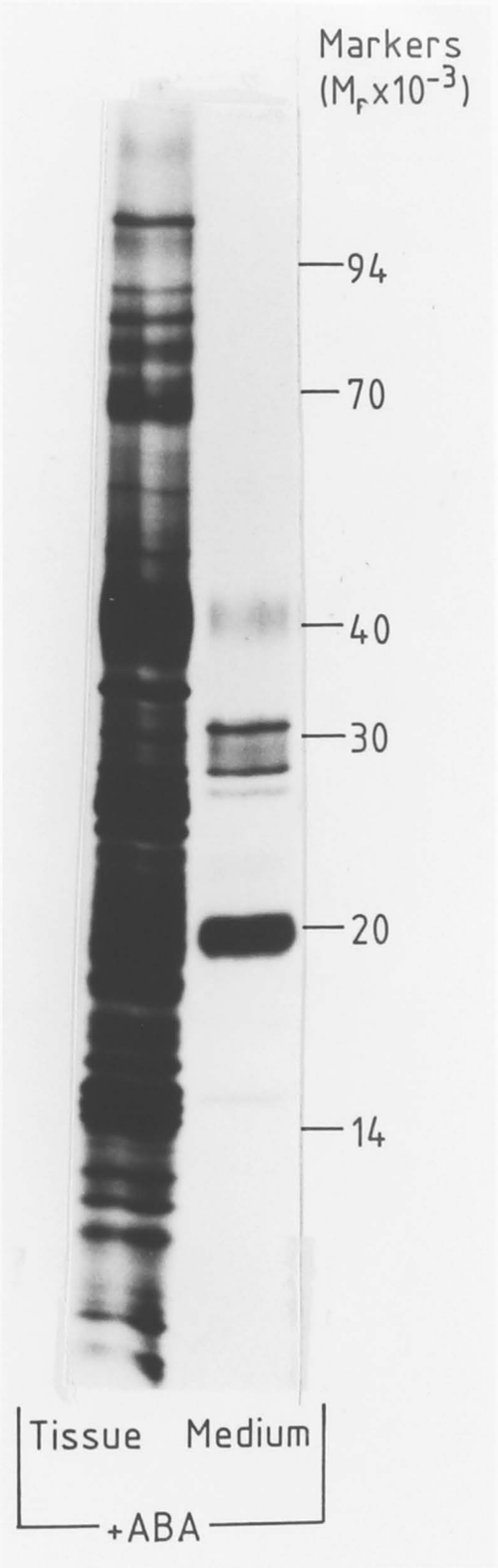
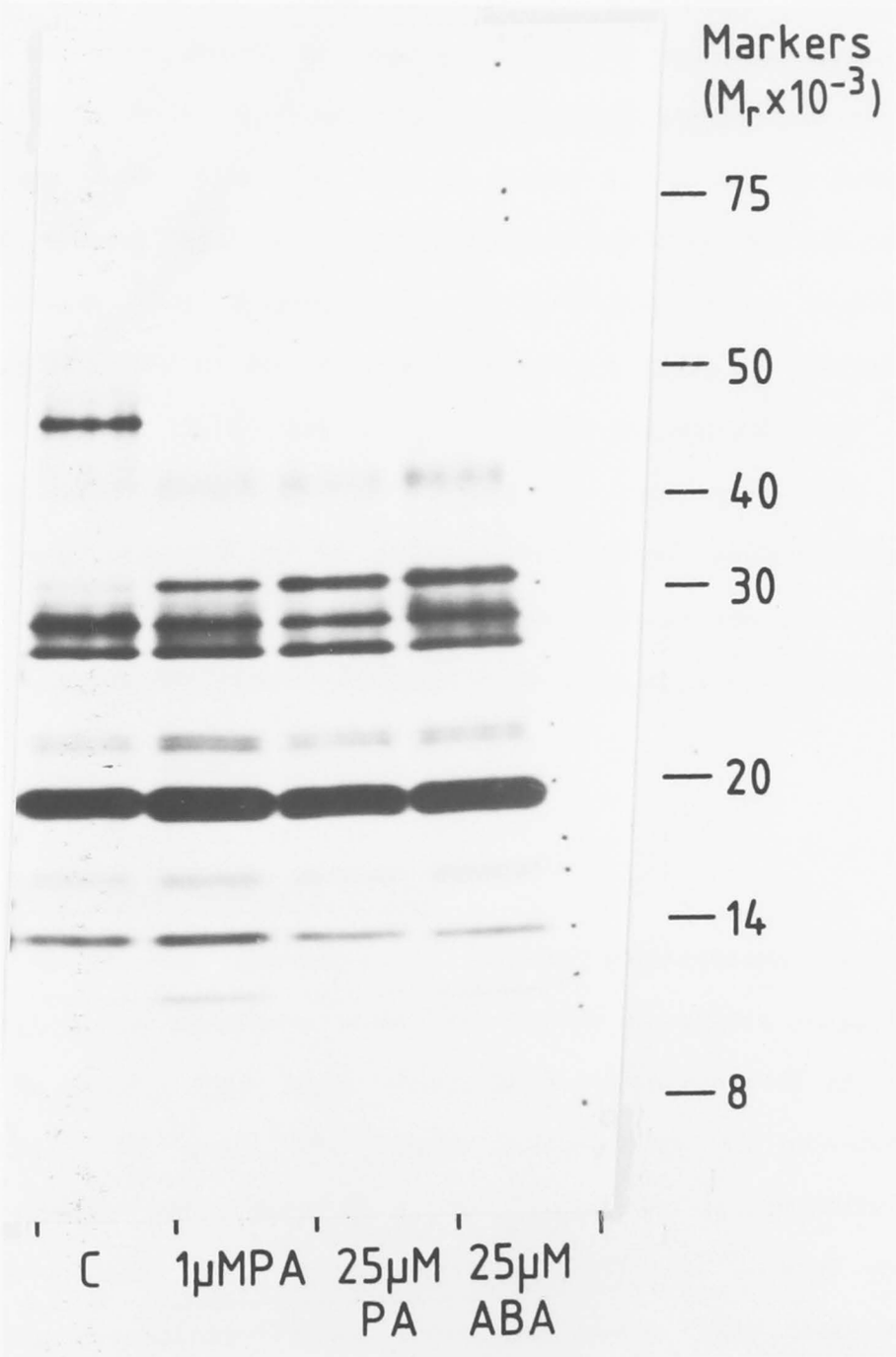

Markers
(M_rx10^{-3})
94
70
40
30
20
14
Tissue
Medium
+ABA

Figure 1.8 Fluorogram of an SDS-polyacrylamide gel of secreted proteins induced by ABA and PA. Aleurone layers were incubated without hormone (control), 1 μM PA, 25 μM PA and 25 μM ABA and and pulse-labelled with [^{35}S]methionine during the last 6 h of a 48 h incubation. Each track contains 1 aleurone layer equivalents of non-heated proteins extracted from the medium. Numbers ($M_r \times 10^{-3}$) indicate positions of protein mobility markers.


Markers
(M_r x 10^{-3})
75
50
40
30
20
14
8
C
1μMPA
25μM PA
25μM ABA

secreted by control layers may sometimes be lower than that of ABA-treated layers which therefore leads to some uncertainty as to whether the $M_r \sim 20,000$ polypeptide is induced by ABA and PA.

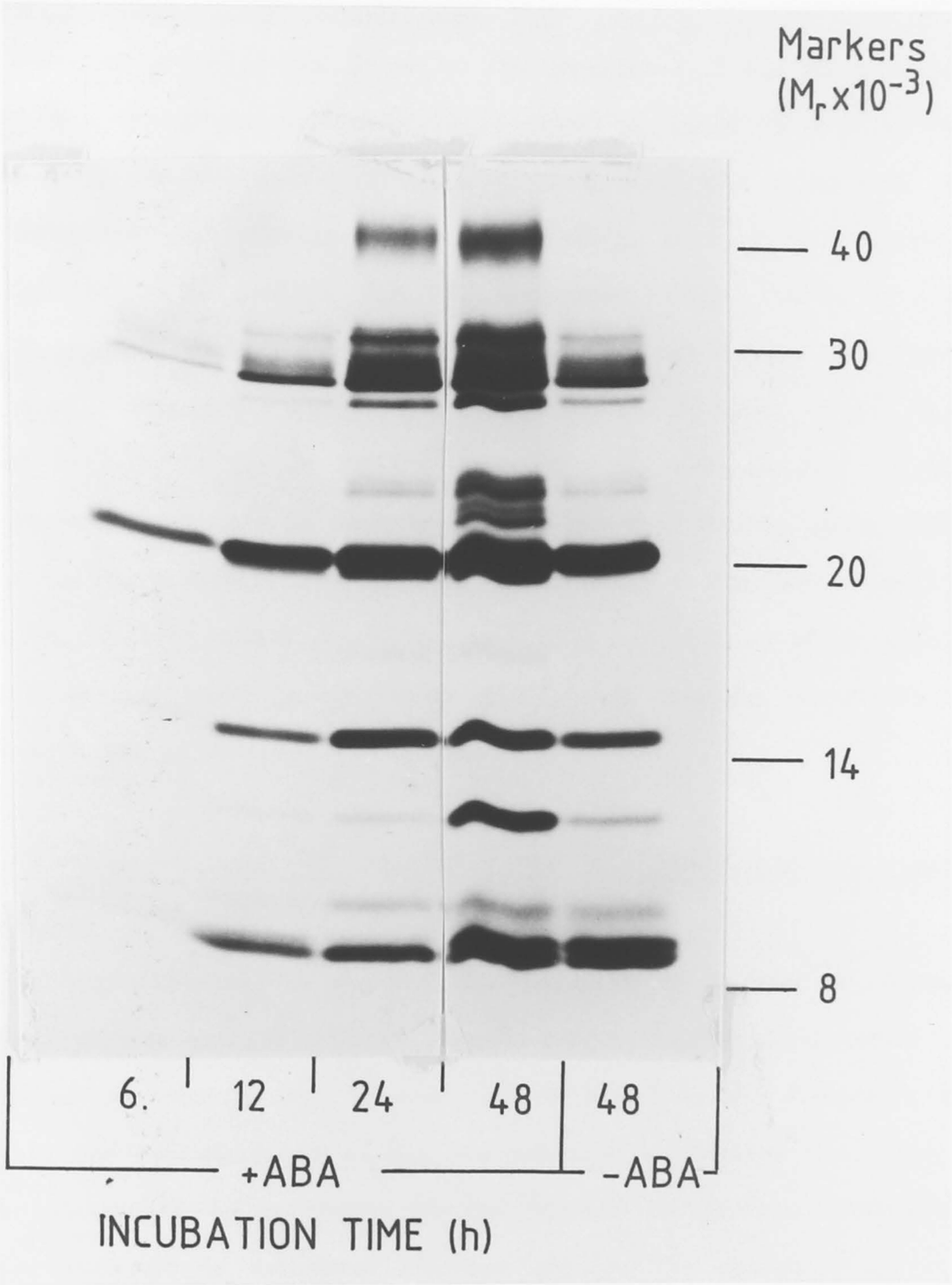
The time-course data (Figure 1.9) for ABA-induced polypeptides which were secreted showed increasing levels for each polypeptide in the medium up to 48 h. By comparing the secreted polypeptide profiles for [-ABA] and [+ABA] tracks at 48 h in Figure 1.9 it can be seen that there were several additional polypeptides secreted by ABA-treated aleurone layers. Two polypeptides, $M_r \sim 40,000$ and $\sim 30,000$ are consistently observed to be secreted. However, a group of polypeptides at $M_r \sim 20,000$ to 22,000 and a $M_r \sim 12,000$ polypeptide seen to be secreted by ABA-treated aleurone layers in this experiment, have previously been observed to be secreted by control layers (data not shown). In conclusion, some of the ABA-induced polypeptides were secreted and similar conclusions were applicable to PA-induced polypeptides.

DISCUSSION

Regulation of Protein Synthesis by ABA

ABA induced the synthesis of several polypeptides in barley aleurone layers, in agreement with the results of others (Higgins *et al.*, 1982, Ho *et al.*, 1985; Mundy 1984). It has been observed that there were at least 20 major ABA-induced polypeptides as revealed by SDS-polyacrylamide gels. Many of these ABA-induced polypeptides were retained in solution after heat-treatement (70°C for 10 min) whereas most proteins precipitated under these conditions. This observation, originally made by J.V. Jacobsen, made the difference between the polypeptide synthesis profiles of control and ABA-treated aleurone layers

Figure 1.9 Fluorogram of an SDS-polyacrylamide gel showing the time-course for protein secretion by aleurone layers, in the absence (-ABA) and presence of 25 μM ABA. Aleurone layers were incubated without or with 25 μM ABA, for different lengths of time and pulse-labelled with [^{35}S]methionine during the last 6 h. Secreted proteins were extracted from the incubation medium. Each track was loaded to represent protein secreted by 5 aleurone layers. Numbers (M_r x 10^{-3}) indicate positions of protein mobility markers.


Markers
(M_rx10^{-3})
40
30
20
14
8
6.
12
24
48
48
+ABA
-ABA
INCUBATION TIME (h)

much clearer than seen in previous studies (Higgins *et al.*, 1982; Ho *et al.*, 1985; Mundy, 1984).

Induction Kinetics of 'ABA Polypeptides'

There was no observable trend in the appearance of the ABA-induced polypeptides. Induction of synthesis occurred as early as 6 h or as late as 24 h and the synthesis declined soon after the appearance of a polypeptide or did continue for at least 48 h. At early time points of incubation (6 h) without ABA, the synthesis of low levels of the ABA polypeptides may be correlated with residual ABA levels which had accumulated during grain development (Goldbach and Michael, 1976). The observed decline in levels of synthesis of 'ABA polypeptides' at 48 h in control layers may be explained by an increase in the amino acid pool due to the presence of endogenous GA_3. As shown in the next chapter, developing aleurone could accumulate specific ABA-induced mRNAs which may still be functional in the mature grain, i.e. they are translatable after imbibition of water by the grains.

Threshold Concentration of ABA Required for Induction of 'ABA Polypeptides'

The concentration of applied ABA required to produce a change in the polypeptide profile from that of the control to the characteristic ABA polypeptide profile was between 0.1 µM and 0.33 µM. Moreover, a relatively low concentration of GA_3 was required to reverse the effect of ABA, as judged by a return to the pattern of protein synthesis normally exhibited by untreated aleurone layers. The concentration of ABA and GA_3 able to induce changes in protein synthesis would be expected

to be due to both the concentration of applied hormone taken up into the tissue and the endogenous hormone concentration. The endogenous ABA concentration has been calculated to be approximately 0.4 μM (Uknes and Ho, 1984), however, it also has to be borne in mind that a certain proportion of the ABA molecules may be compartmentalized and not uniformly distributed within the cellular compartment.

Induction of 'ABA Polypeptides' by PA

Phaseic acid was at least as active as ABA in inducing the synthesis of the ABA polypeptides, in contrast to the results of Ho *et al.* (1985). PA was also as effective as ABA in the inhibition of α-amylase synthesis (Dashek *et al.*, 1979) and in stomatal closure in several plant species (Sharkey and Raschke, 1980). Therefore, it is unlikely that the physiological activity of ABA is decreased by oxidation to PA. However, the activity of ABA and PA may be reduced when PA is metabolised further to DPA which was shown to be inactive in several bioassays (Dashek *et al.*, 1979, and Sharkey and Raschke, 1980).

Secretion of 'ABA Polypeptides'

At least two (groups) of the many ABA-induced polypeptides were secreted. No difference was observed in the pattern of secreted polypeptides between ABA and PA-treated aleurone layers. Although Higgins *et al.* (1982) interpreted their data to mean that none of the ABA-induced polypeptides were secreted their polypeptide profile for secreted polypeptides (in Figure 5B) in the presence of 25 μM ABA did in fact reveal a new band in the ABA track which closely corresponded with our diffuse band at $M_r \sim$ 40,000. The level of each polypeptide that was

secreted by ABA-incubated aleurone into the medium increased with time and the absence of at least two groups of polypeptides in control medium indicated that the polypeptides were probably actively secreted into the medium and not due to leakage through the cell membrane. The low proportion of polypeptides observed to be secreted into the medium may be explained by the lag time for an aleurone polypeptide to be secreted. In order to obtain a more accurate quantitative representation on secreted polypeptides, a longer time of labelling may be appropriate, followed by a chase with cold methionine.

SUMMARY

In barley aleurone layers, ABA induces the synthesis of at least 20 major polypeptides, in the size range of $M_r \sim 10,000$ to 102,000.

'ABA polypeptides' exhibit different kinetics of induction following ABA treatment. Differences were also seen in the duration of synthesis in response to ABA.

The induction of 'ABA polypeptides' requires the presence of a threshold concentration of exogenous ABA between 0.1 μM and 0.3 μM.

The effect of 1 μM ABA is reversed by the simultaneous addition of GA_3 as evident from the difference in the patterns of protein synthesis when aleurone layers were incubated in 1 μM ABA alone or in 1 μM ABA + 0.05 μM GA_3.

The activity of PA is virtually the same as that of ABA in inducing synthesis of 'ABA polypeptides' and in the suppression of α-amylase synthesis.

Particular PA- and ABA-induced polypeptides (a low proportion of the total induced polypeptides) are secreted.

Most of the PA- and ABA-induced polypeptides remained in solution after heat treatment.

CHAPTER 2

INTRODUCTION

In Chapter 1, I presented results on the induction of new polypeptides by ABA and its metabolite, PA. The time-course data for appearance of 'ABA polypeptides' clearly indicated that these polypeptides were synthesised with different lag periods after exposure to ABA. In addition, once synthesis began, a particular polypeptide could continue synthesis for a short or long period prior to its decline. Since these 'ABA polypeptides' were detected only after a certain incubation time, it was most likely that synthesis and decline of 'ABA polypeptides' were controlled through regulation of gene expression. Therefore, following observations made in Chapter 1, further studies were conducted to test if changes in protein synthesis were due to mechanisms involving changes in gene expression. By analogy with α-amylase synthesis, it is possible that the regulation of 'ABA polypeptides' may be exerted at the level of mRNA abundance.

Several techniques are available to assay for changes in levels of mRNA in cells. One of these employs the cell-free translation of total RNAs i.e. all translatable exogenous mRNAs are translated by ribosomes (of wheat germ or rabbit reticulocytes) into polypeptides which can then be resolved on a gel. Any changes in the spectrum of polypeptides (i.e. the in vitro translation products) programmed by exogenous total RNAs reflect changes in abundance of mRNAs.

Alternatively, changes in mRNA abundance could also be assayed by probing for specific mRNA levels with homologous sequences e.g. by

using a complementary DNA (cDNA) strand. Detection of homologous mRNA by its complementary DNA depends on homology of sequences only and is unaffected by the translability of that message.

cDNA clones have been constructed (by P.M. Chandler) from an aleurone layer RNA preparation enriched for ABA-induced mRNA i.e. total RNA isolated from ABA-incubated aleurone layers as opposed to those from [control] aleurone layers which would contain low levels or none of the ABA-induced mRNAs. These cDNA clones would be radiolabelled and used as probes to measure any relative changes in mRNA levels that may be promoted or suppressed by ABA.

A technique used to detect DNA-RNA hybridization, called a Northern blot, is done by fractionating the different mRNAs by electrophoresis and blotting them onto a filter membrane. The radiolabelled cDNA probes are then hybridized to the immobilised RNA. When the probes have been removed, the blots are then exposed to X-ray film. The degree of hybridization between an mRNA and the radioactive probe is monitored by the intensity of band(s) (on X-ray film) at a position which identifies a specific mRNA.

In the aleurone system, changes in mRNA abundance have been shown to be a factor in bringing about changes in protein synthesis. Higgins *et al.* (1982), Muthukrishnan *et al.* (1983) and Mundy *et al.* (1986) employed cell-free translation to show that the synthesis of α-amylase in barley aleurone layers is regulated by changes in translatable α-amylase mRNA levels, and that ABA suppressed the GA_3-mediated induction of α-amylase mRNA.

Other researchers have arrived at the same conclusion by doing Northern blots. Muthukrishnan *et al.*, (1983), Rogers and Milliman (1983)

and Chandler *et al.*, (1984) used α-amylase cDNA probes to hybridize with RNA isolated from aleurone layers that had been incubated with ABA and GA_3. They concluded that ABA and GA_3 regulate α-amylase protein synthesis by modulating α-amylase mRNA abundance i.e. by transcriptional control or by a change in the rate of turnover of mRNAs. However, since then, run off transcription studies (study of gene transcription in plant nuclei) by Zwar and Hooley (1986) and Jacobsen and Beach (1985) provided evidence that GA_3 and ABA regulate the transcription of α-amylase.

Identity of GA_3- and ABA-induced polypeptides

The GA_3-promoted proteins have been identified as hydrolytic enzymes which are important during germination. Enzymes such as proteases and α-amylases hydrolyse starch and proteins to sugars and amino acids which are necessary for growth of the embryo.

Mundy (1984) identified one of the 'ABA polypeptides' as the bifunctional α-amylase/subtilisin inhibitor (ASI), and its amino acid sequence is now known (Svendsen *et al.*, 1986). ASI mRNA levels are regulated in the opposite way to that of α-amylase mRNA i.e. the level of ASI mRNA was promoted by ABA, but suppressed by GA_3 as shown by cell-free translation and immunoprecipitation with antibodies to ASI (Mundy *et al.*, 1986).

Identification of ASI has led to speculations on its function in the barley grain. Although ASI mRNA is detectable in aleurone of mature barley grains, it is not detectable in aleurone of developing barley grains (Mundy *et al.*, 1986). Perhaps, ASI is present in aleurone of mature grains where it plays a part in the prevention of precocious germination.

With the exception of ASI, the functions of the other ABA-induced proteins (more than 20 major polypeptides) are unknown. Knowing their functions may help towards the understanding of ABA action. It is possible to know the function of an uncharacterized protein if its amino acid sequence is known and if the sequence is homologous with that of another known protein(s).

At this stage, it is possible to sequence the DNA of ABA-induced cDNA clones. From the nucleotide sequence, an amino acid sequence can be deduced for comparison with sequences of known proteins in the Data Bank so that the function of the protein (corresponding to a particular clone) may be elucidated. It was hoped that one of the ABA-induced cDNA clones corresponded to the ASI gene.

Increase in Endogenous ABA Levels in Developing Barley Grains

The aleurone system of barley was intially used because it was possible to apply GA_3 to it in order to mimic processes that occur during germination. GA_3 is known to be released by the embryo, after which it exerts its effect through its action on cells of the aleurone layer.

ABA, however, is known to promote dormancy in plants and seeds. Obviously, application of ABA to a mature aleurone layer is a somewhat artificial situation since during germination, the presence of ABA may negate the effects of GA_3 (Higgins *et al.*, 1982). Morever, knowing that one of the ABA-induced proteins is an α-amylase/subtilisin inhibitor, its presence may reduce the activity of α-amylase (high pI only).

Therefore, it is of interest to study a situation when a plant encounters increased ABA levels, sufficient to bring about changes such as those to be seen in aleurone of mature grains.

Goldbach and Michael (1976) showed that the level of endogenous ABA in developing barley grains is low in the first few days after pollination, and increases to reach a maximum level at the same time as maximum fresh weight of the grains (Goldbach and Michael, 1976). The stimulus for the increase in endogenous ABA levels in developing barley grains is not known although genetic and environmental factors may influence the ABA content considerably. It is also unknown whether the aleurone layers of developing barley grains are responsive to the increasing endogenous ABA levels which would be manifested as increased abundance of specific ABA-induced mRNAs. As mentioned above, Mundy *et al.* (1986) did not detect any ASI mRNA in developing aleurone layers.

In this Chapter, data are presented on the regulation of mRNA abundance by ABA and PA to complement results of *in vivo* pulse-labelling studies presented in Chapter 1. *In vitro* translation (employing both or either one of the cell-free translation systems: wheat germ and rabbit reticulocyte) was used to study changes in mRNA abundance and the *in vitro* products were examined for co-mobility with *in vivo* pulse-labelled polypeptides, and retention in solution after heat treatment as was demonstrated for some of the *in vivo* 'ABA polypeptides' as shown in Chapter 1. Northern hybridization analyses were also done to support and extend *in vivo* and *in vitro* studies. In addition, changes in the levels of ABA-induced mRNAs were studied in aleurone layers of developing barley grains (by Northern hybridizations). One of the ABA-induced cDNA clones (which had also been used as a hybridization probe) was sequenced. Its amino acid sequence was deduced (from the nucleotide sequence) for comparison with sequences of known proteins in the Data Bank.

MATERIALS AND METHODS

Isolation of Total RNA from Aleruone Layers

Aleurone layers were incubated as described in Methods for Chapter 1. Total RNA isolation was by the methods of Higgins *et al.* (1976), with modifications. The layers were ground for 2 min in 10 ml of buffer containing 0.1 M sodium glycinate, pH 9.5, 0.01 M EDTA, 0.1 M sodium chloride and 1% SDS, with 1% insoluble polyvinylpyrrolidone (PVP), 50 mg bentonite and 1% Na-deoxycholate and warmed at 37°C for 5 min. After centrifugation at 10,000 x g for 10 min the supernatant was transfered to a large test-tube. The pellet was re-extracted with the same buffer, combined with the first supernatant and extracted with an equal volume of phenol-chloroform-isoamylalcohol (50:48:2). The phases were separated by centrifugation at 10,000 x g for 10 min.

The RNA in the aqueous phase was precipitated by addition of 2.5 volumes of ethanol and stored overnight at -20°C. It was then pelleted by centrifugation, redissolved in 3 ml of 0.5 M sodium acetate, pH 5.8 and precipitated with 0.4 ml of 1% cetyltrimethylammonium bromide for 1 h at 0°C followed by centrifugation and washed with 0.1 M sodium acetate (pH 5.8) in 70% ethanol. The pellet was dissolved in 100 1 H_2O and precipitated by addition of 1 ml of 3 M sodium acetate (pH 5.8) containing 5 mM EDTA, and left on ice for 3 h. After centrifugation, the pellet was washed in 3 M sodium acetate (pH 5.8) and finally with 70% ethanol. The pellet was dried and dissolved in 100 1 H_2O. Poly(A) RNA was obtained by chromatography of total RNA on oligo dT cellulose columns (Type 3, Collaborative Research, Massachusetts) following the manufacturer's instructions.

Isolation of Total RNA from Aleurone Layers of Developing Grain

Barley (*Hordeum vulgare* c.v. Himalaya, 1984 grains obtained from J.V. Jacobsen, CSIRO Division of Plant Industry, Canberra) plants were grown in a phytotron glasshouse under natural light at 18°C/13°C day/night temperatures. Awns were tagged when emerged from the flag leaves, and grains were obtained at 20 to 60 days after awn emergence (DAE), weighed, aleurone layers removed from the starchy endosperm and total RNA isolated as above. Isolation of total RNA from developing aleurone layers at 60 DAE was unsuccessful, while earlier DAE grains gave low yields of total RNA.

In Vitro Translation in a Wheat Germ System

All components used with system RPN.1 and protocol were obtained from Amersham, UK. The optimum concentration of potassium ions was determined from a series of incubations each of which had an identical quantity of premix but a varying concentration of potassium acetate. The optimum mRNA concentration was determined by adding a range of concentrations of total RNA (5 to 25 µg) to the premix. Each 30 µl reaction contained either no added RNA for control, 10 µg of [-ABA] or [+ABA] total RNA, 50% v/v wheat germ extract, a final concentration of 60 µM cold amino acid mix minus methionine (Amersham, UK), 34 mM KOAc, 18.5 mBq/µl [^{35}S]methionine (specific activity approximately 51 TBq/mmol).

The components were mixed by gentle agitation, incubated at 25°C for 1 h, then at 37°C for 10 mins to hydrolyse aminoacyl-tRNA complexes.

For loading equal TCA precipitable radioactivity on SDS-polyacrylamide gels, 2 µl of each reaction was assayed for TCA-precipitable counts on 3 mm Whatman paper. The appropriate volume of translation reaction was prepared for electrophoresis as for in vivo pulse-labelled products.

In one case, an SDS-polyacrylamide gel was loaded for equal volumes of incubation mixes instead of equal TCA precipitable radioactivity. To obtain heat-treated in vitro translation products, 4 µl of the incubation mix was heated in 50 µl of protein extraction buffer and prepared for electrophoresis as described for *in vivo* pulse-labelled products.

In Vitro Translation in a Rabbit Reticulocyte System

All components of the rabbit reticulocyte lysate (N.90) and protocol were obtained from Amersham, UK. Each 30 µl reaction contained 80% v/v lysate, 55.5 mBq/µl [^{35}S]methionine, 5 µM EGTA, 0.4 units/µl human placental ribonuclease inhibitor and 10-20 µg of total RNA.

Reactions were incubated at 30°C for 1 h, transferred to 37°C for 10 min to hydrolyse aminoacyl-tRNA complexes, and kept on ice. TCA-precipitable cpm were determined for 3 µl of incubation mix.

For non-heated products: 100,000 TCA-precipitable cpm were prepared for electrophoresis, as described for *in vivo* pulse-labelled polypeptides (Chapter 1 Methods). For heated products, 200,000 TCA-precipitable cpm was heated at 70°C for 10 min. in 50 µl of protein extraction buffer and prepared for electrophoresis, as for heated in vivo pulse-labelled polypeptides (Chapter 1, Methods).

Colony Hybridization

Transformed cells, screened for tetracyline resistance and ampicillin sensitivity were grown in media contained in microtitre trays. The cells were transferred in duplicates onto nitrocellulose filters placed on agar media and grown at 30°C overnight. The colonies on the plates were subjected to 10% SDS for 25 min, denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 15 min and neutralising solution (1.5 M NaCl, 1 M Tris.Cl pH8) for 15 min. The filters were air-dried, then baked at 80°C in a vacuum oven for 2 h, and pre-washed twice over 12-18 h in 6XSSC, 0.1% SDS. Subsequently, the colony filters were pre-hybridized etc. as for Northern filters (nitrocellulose) as previously described by Chandler *et al.*, (1983).

Filters were prehybridized at 42°C in 50% formamide, 3 x SSC (SSC=0.15 M NaCl, 15 mM trisodium citrate), 10 x Denhardt's solution (in w/v, 0.2% BSA, 0.2% PVP, 0.2% Ficoll 400; Pharmacia), 0.1% SDS, 25 µg/ml denatured calf thymus DNA, 10 µg/ml poly(A), for 6 to 18 h, and hybridized under the same conditions with approximately 5 x 10^6 cpm ^{32}P-labelled DNA for approximately 24 h. After hybridization, filters were washed in 2 x SSC containing 0.1% SDS at 70°C for 2 h, then 0.1 x SSC, 0.1% SDS at 50°C for 1 h before autoradiography with an intensifying screen.

Preparation of cDNA Probes

Poly(A) RNAs (0.5 µg - 1 µg) isolated from [-ABA] and [+ABA] aleurone layers, were used as templates in 100 µl reactions containing 50 mM Tris, 80 mM KCl (pH 8.3), 10 mM $MgCl_2$, 10 mM DTT, 10 units oligo

dT, 500 µM each of dATP, dGTP, TTP; 10 µM dCTP, 80 units RNase inhibitor, 4 mM Na-pyrophosphate (pH 7.8), 70 units reverse transcriptase, 1.85 mBq [α-^{32}P]dATP.

After incubation at 41°C for 1 h, RNA template was hydrolysed by addition of 50 µl of 1 M NaOH at 60°C for 1 h, neutralised with 50 µl of 2.5 M Tris-HCl and extracted with buffer-saturated phenol. The cDNA probe was loaded onto a G-50 Sephadex column and fractions containing the excluded material were peak pooled, freeze-dried and disolved in 10 ml of hybridization buffer (as in colony hybridization).

Preparation of Northern Blots

Samples of total RNA (10 µg) were denatured in 50 µl volumes (containing 5 µl of 10 X MOPS buffer (=0.2 M MOPS, 0.05 M Na-acetate, 0.01 M EDTA pH7), 25 µl formamide, 9 µl formaldehyde) at 70°C for 5 min and fractionated by electrophoresis in a 1.5% agarose-formaldehyde (6.5% v/v) gel in 1 X MOPS buffer. The RNA was transferred to either Hybond-nylon or nitrocellulose filters overnight in 20 X SSC (SSC=0.15 M NaCl, 15 mM trisodium citrate). To fix RNA, Hybond-nylon filters were exposed to UV light for 5 mins, whereas the nitrocellulose filters were baked at 80°C for 2 h. The filters were pre-hybridized and hybridized to radioactive inserts of cDNA clones under the same conditions as colony filters for colony hybridization.

Oligo-Labelling of Insert DNA

Radiolabelling of DNA was done using the BRESA oligo-labelling kit, following the manufacturer's instructions. Insert DNA (100 ng) was heat-denatured and added to the nucleotide/buffer cocktail containing 1.85 mBq [α-^{32}P]dCTP, incubated at 40°C for 20 min and ethanol precipitated.

Densitometry

Relative mRNA levels for pHV A34 (Fig. 2.7b) and pHV A39 (Fig. 2.7d) in [-ABA] and [+ABA] total RNA were estimated by densitometry of the X-ray film and comparison of peak areas by weighing.

Sequencing of pHV A34

DNA was sequenced in both strands of pHV A34 (after subcloning into pUC 19) by the chemical sequencing method of Maxam and Gilbert (1980), using the G, G+A, T+C, and C reactions. The sequencing strategy is shown in Fig. 2.12. Entire nucleotide sequences were determined by sequencing of 5' or 3' radiolabelled restriction endonuclease fragments.

RESULTS

Cell-free Translation Studies in the Wheat Germ System

ABA-induced Changes in mRNA Abundance

In Chapter 1, it was shown that there was a lag time of 6 h to 24 h before the appearance of novel polypeptides after treatment of aleurone layers with ABA. It was suggested that the changes seen would most probably involve changes in gene expression. Possible changes in mRNA levels in aleurone layers treated with ABA were investigated by cell-free translation of total RNA isolated from aleurone layers incubated for 16 h without [-ABA] and with 25 μM ABA [+ABA].

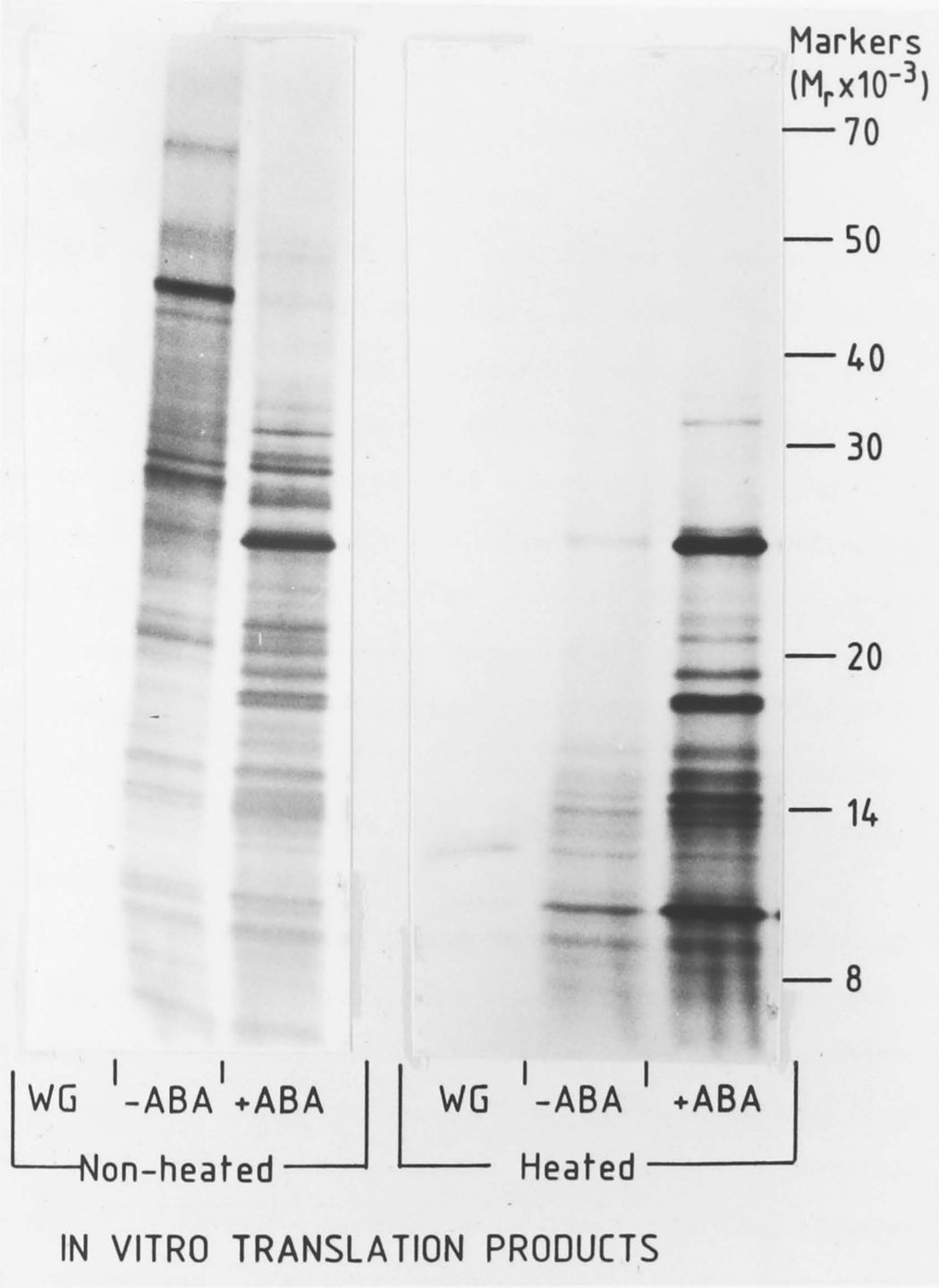
Figure 2.1 shows that compared with the control (WG), addition of RNA ([-ABA] and [+ABA] tracks) resulted in stimulation of polypeptide synthesis in vitro. The patterns of polypeptide synthesis (see non-heated tracks) for [-ABA] and [+ABA] total RNA were different, indicating that ABA-induced changes in protein synthesis as seen in Chapter 1 reflected differences in the levels of translatable mRNAs, in agreement with Higgins *et al.*,(1982) and Mundy *et al.*,(1986). In the [-ABA] track, α-amylase ($M_r \sim 46,000$) was the most abundant polypeptide synthesised from RNA of control aleurone.

When the translation products were subjected to the same heating regime (70°C for 10 min) as for *in vivo* pulse-labelled polypeptides (e.g. Figure 1.1), α-amylase and most other polypeptides synthesised from [-ABA] mRNA were precipitated from solution (see 'heated' tracks). In contrast, many of the polypeptides synthesised from [+ABA] mRNA were retained in solution. The major ABA-induced polypeptides in the heated profile (e.g. $M_r \sim 32,000$, 25,000 and 17,000)

Figure 2.1 Fluorogram of an SDS-polyacrylamide gel of wheat germ *in vitro* translation products made to total RNA isolated from aleurone layers incubated for 16 h without (-ABA) and with 25 µM ABA(+ABA). *In vitro* translation reactions (25 µl) contained no RNA (WG: wheat germ translation products) or 10 µg of the appropriate total RNA and [^{35}S]methionine.

Non-heated series: 2 µl of the incubation mix was loaded per track.

Heated series: 4 µl of incubation mix was made up to 50 µl with extraction buffer heated at 70°C for 10 min and prepared for loading as in Methods.



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co-migrated with polypeptides in the non-heated profile. This suggested that some of the *in vitro* translation products of the ABA-induced mRNAs were soluble after heating, as found for the ABA-induced *in vivo* pulse-labelled polypeptides.

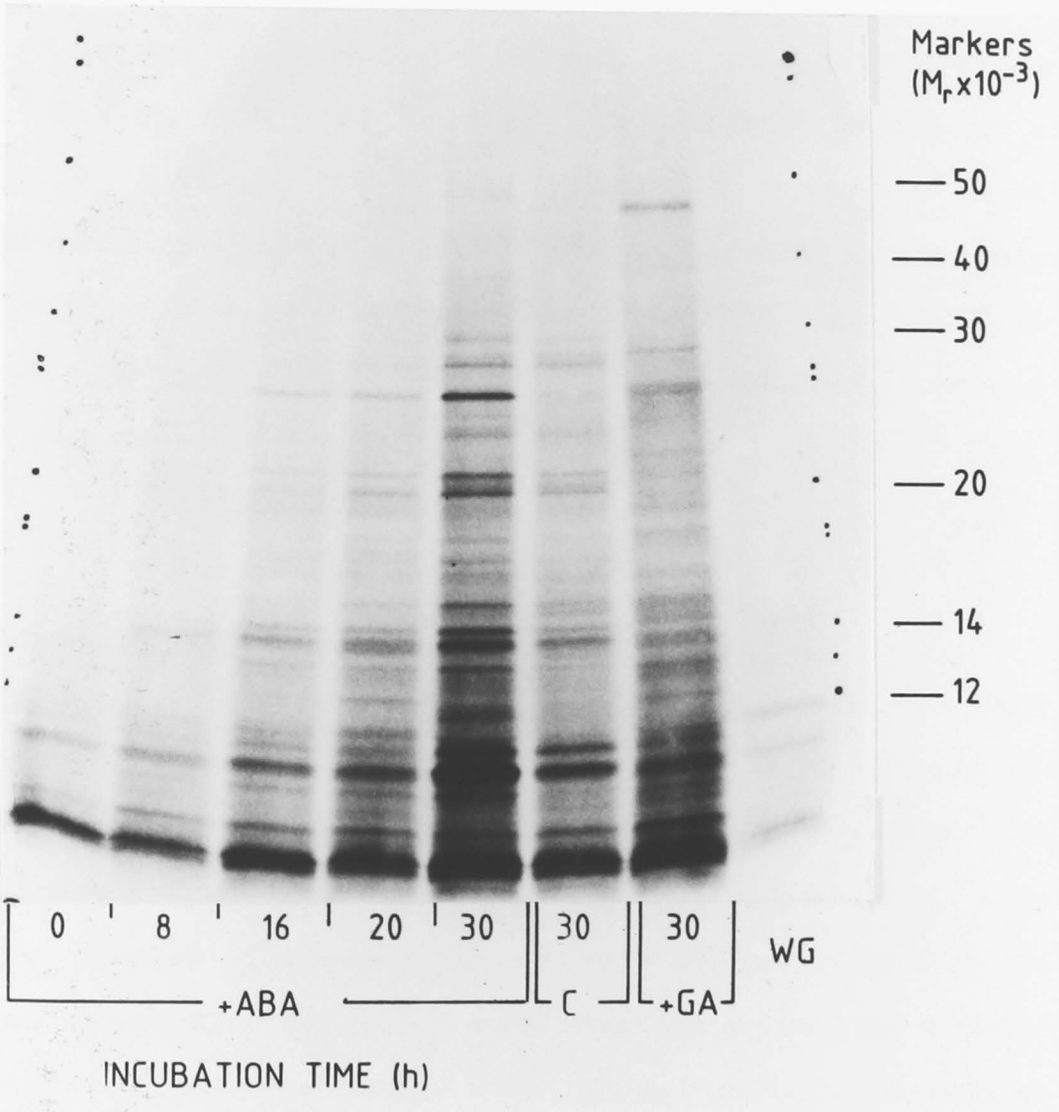
Time-course Studies

The time-course for novel polypeptides to be seen by *in vivo* pulse-labelling was shown in Figure 1.2 and I wanted to determine whether those changes in levels of ABA-induced polypeptides were related to changes in mRNA levels as judged by in vitro translation products.

Figure 2.2 shows the profile of polypeptides whose synthesis was programmed by total RNAs isolated from aleurone layers incubated in 25 μM ABA for 0 h to 30 h. Control (C) layers and GA_3-treated layers were incubated for 30 h. These *in vitro* translation products were not subjected to the 70°C heat treatment. Between 0 h and 30 h, there were no obvious changes in the levels of synthesis for particular polypeptides which were programmed by [+ABA] total RNA. The polypeptides profiles obtained for the time-course *in vitro* translation studies did not indicate specific times of induction for ABA-induced mRNAs.

The difference in intensities of the polypeptide profiles (in each track) was partly due to the loading of equal volumes of translation reactions, instead of loading for equal TCA-precipitable cpm. However, similar results were obtained when poly(A)$^+$ RNAs (isolated from time-course total RNAs which programmed the *in vitro* translation reactions in Fig. 2.2) were translated in a rabbit reticulocyte system and the translation reactions subsequently loaded for equal TCA-precipitable cpm. These observations indicated that there may be

Figure 2.2 Fluorogram of an SDS-polyacrylamide gel. *In vitro* translation products of ABA time-course RNAs. Aleurone layers were incubated in 25 μM ABA for 0, 8, 16, 20 and 30 h; without hormone for 30 h (control); or with 1 μM GA_3 for 30 h. Total RNAs isolated from these layers were translated in a wheat germ cell-free system. WG: products of endogenous wheat germ mRNAs. Each track was loaded for equal volumes of translation reaction.


Markers
(M_rx10^{-3})
50
40
30
20
14
12
0
8
16
20
30
30
30
WG
+ABA
C
+GA
INCUBATION TIME (h)

differences in translatability of the total RNA samples isolated from aleurone layers incubated for different durations with or without plant hormones.

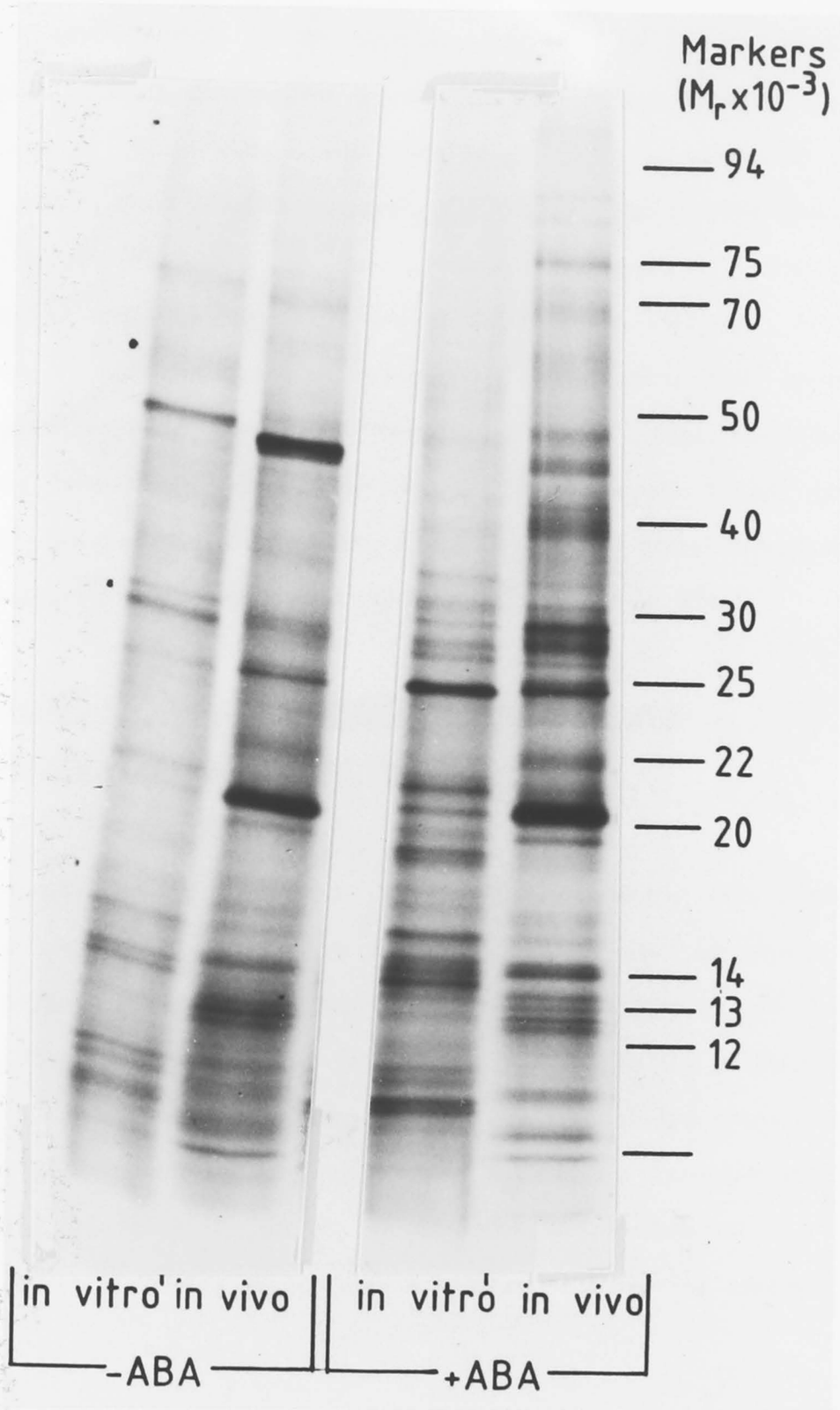
Comparison of the polypeptide profiles of *in vitro* translation products programmed by mRNAs isolated from aleurone layers incubated for 30 h in 25 μM ABA, control or 1 μM GA_3 showed that there was a large difference between the translation products of [+ABA], [Control] and [+GA] total RNAs. Only [+GA] total RNA programmed the translation of α-amylase, M_r ∿ 46,000. The *in vitro* translation products of [+ABA] total RNA exhibited a relatively greater abundance of mRNA species which programmed the synthesis of polypeptides, M_r ∿ 25,000 and 13,000. In the WG track, several polypeptides of low M_r were synthesised in response to wheat germ endogenous RNAs.

Comparison of *In Vitro* and *In Vivo* Products

Higgins *et al.* (1982) showed that α-amylase synthesised in a wheat germ *in vitro* translation system, had an M_r of ∿ 46,000 whereas the *in vivo* pulse-labelled polypeptide had a lower M_r of ∿ 44,000. The *in vitro* translation product was larger than that synthesised *in vivo* due to the presence of a signal peptide which was not removed by the wheat germ preparation. Figure 2.3 shows that the polypeptide profiles for *in vitro* (translation) and *in vivo* (pulse-labelled) polypeptides were different for the [-ABA] aleurone layers. The *in vitro* translation product of α-amylase at M_r ∿ 46,000 was larger than the *in vivo* pulse-labelled α-amylase at M_r ∿ 44,000.

In the case of the [+ABA] aleurone layers, although the *in vitro* and *in vivo* profiles were generally different several bands co-migrated,

Figure 2.3 Fluorogram of an SDS-polyacrylamide gel. Comparison of *in vitro* translation products with *in vivo* pulse-labelled polypeptides (non-heated samples). Preparation of *in vitro* translation products: Total RNAs were isolated from aleurone layers incubated for 16 h without hormone (-ABA) and with 25 µM ABA(+ABA) and translated in a wheat germ translation system. Preparation of *in vivo* pulse-labelled polypeptides: Aleurone layers were incubated for 16 h without hormone (-ABA) and with 25 µM ABA(+ABA) and pulse-labelled with [^{35}S]methionine during the last 2 h of incubation.


Markers
(M_r x 10^{-3})
94
75
70
50
40
30
25
22
20
14
13
12
in vitro
in vivo
in vitro
in vivo
-ABA
+ABA

with M_r between 25,000 to 30,000. However, it was not certain if the co-migration bands represented the same polypeptides.

The largest *in vitro* translation products of $M_r \sim$ 70,000 (observed in the [-ABA] track) synthesised in the wheat germ system were not as large as some of the *in vivo* pulse-labelled polypeptides which may have an M_r of greter than 100,000 (largest ABA-induced polypeptide seen by pulse-labelling). This may be due to the occurrence of premature termination in the wheat germ cell-free translation system, i.e. the translation of polypeptides was prematurely terminated. This problem was not prevalent in the rabbit recticulocyte cell-free translation system (see below). Therefore, in general the *in vitro* (wheat germ) products of ABA-treated aleurone layers did not have *in vitro* counterparts, with the exception of polypeptides with $M_r \sim$ 25,000 to 30,000.

Cell-free Translation Studies in Rabbit Reticulocyte System

ABA- and PA-induced Changes in mRNA Abundance

For comparison of *in vitro* translation products, the rabbit reticulocyte cell-free translation system was also used to study *in vitro* translation products programmed by Control, [+ABA] and in addition, [+PA] total RNAs. This experiment was designed to compare the polypeptide profiles of *in vitro* translated products of [+ABA] and [+PA] total RNAs, and to test if the polypeptides translated in the reticulocyte system also exhibited the same properties as the ABA- and PA-induced *in vivo* pulse-labelled polypeptides after being subjected to a heating regime.

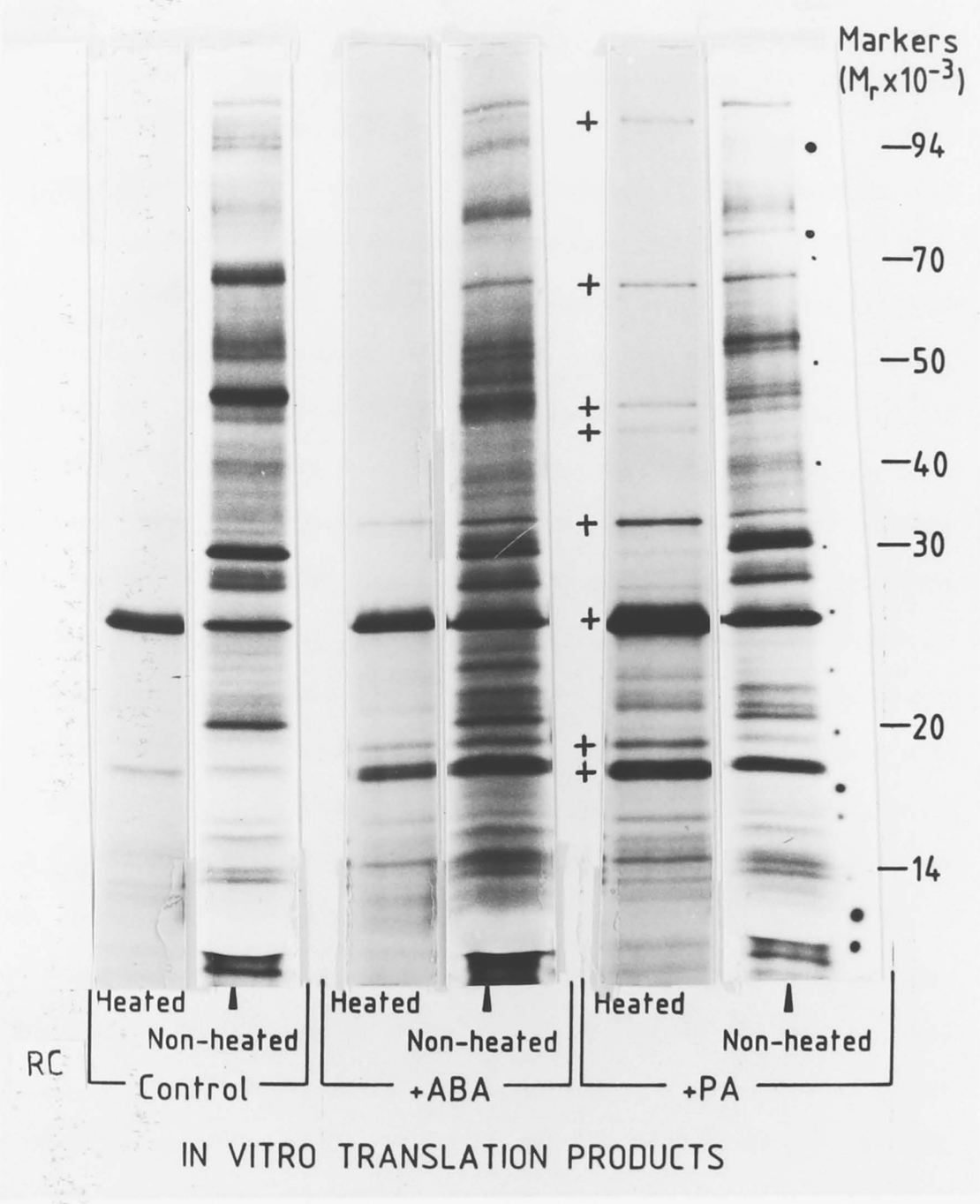
Figure 2.4 shows the reticulocyte *in vitro* translation products of [Control], [+ABA] and [+PA] total RNAs. The non-heated translation products yielded polypeptides in a wide range of M_r from the very low <8,000 (which were not resolved) to $M_r \sim 100,000$ which was comparable with the M_r of *in vivo* polypeptides. This also indicated that premature termination was not a problem in the reticulocyte system. In the non-heated [Control] track, the *in vitro* translation products included $M_r \sim 68,000$ and $M_r \sim 46,000$ which were not translated when [+ABA] and [+PA] total RNAs were added to the translation reaction. The profile for *in vitro* translation products (non-heated) of [+ABA] and [+PA] total RNAs were similar, i.e. ABA and PA regulated the abundance of the same population of mRNAs.

Heat treatment resulted in the precipitation of most of the *in vitro* translation products of [Control] RNA, with the exception of the $M_r \sim 25,000$ polypeptide. This polypeptide was not translated in the wheat germ system when programmed by [Control] RNA, as shown in Fig. 2.1 which was in agreement with the data of Higgins *et al.* (1982). The synthesis of the ABA-induced $M_r \sim 25,000$ polypeptide programmed by [Control] total RNA will be explained in the Discussion.

When *in vitro* translation products of [+ABA] and [+PA] total RNAs were heat treated, several of the polypeptides remained in solution, resulting in similar polypeptide profiles, although there was significant loss of radioactivity (probably during the purification procedure) from the [+ABA] translation products. Although the bands in the heated sample of the in vitro translation products of [+ABA] total RNA are of lighter intensity, they matched, band for band, with the corresponding heated profile of [+PA] total RNA. However, by viewing the heated [+PA] translation products, it is clear that several of the *in vitro* translation products synthesised in the reticulocyte system remained

Figure 2.4 Fluorogram of an SDS-polyacrylamide gel. Comparison of heated and non-heated *in vitro* translation products in the rabbit reticulocyte system. Aleurone layers were incubated without hormone (control), with 25 µM ABA or 25 µM PA for 16 h. Total RNA was isolated and translated in a reticulocyte cell-free translation system in the presence of [^{35}S]methionine. Translation products (200,000 TCA-precipitable cpm) were heated at 70°C for 10 min, then prepared for electrophoresis. Non-heated tracks were loaded for 100,000 TCA-precipitable cpm. Different film exposure times for non-heated and heated tracks were used to optimize visualisation of bands against the high background in the non-heated profile.

RC: reticulocyte products with no added RNA.



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in solution after the heat treatment. The most abundant *in vitro* product of [+ABA] and [+PA] aleurone layers is the $M_r \sim$ 25,000 polypeptide, in agreement with data obtained using the wheat germ system.

Comparison of *In Vitro* and *In Vivo* Products

In order to make a more detailed comparison on the polypeptide profiles of cell-free translation products synthesised in a rabbit reticulocyte system (*in vitro*) and the pulse-labelled polypeptides (*in vivo*), comparisons were made on heated proteins, as shown in Fig. 2.5. *in vitro* polypeptides were prepared as follows: Total RNA was isolated from aleurone layers incubated for 16 h without addition [Control], with 25 µM ABA [+ABA] or with 25 µM PA [+PA], and translated in the presence of [^{35}S]methionine in a reticulocyte cell-free system. *In vitro* polypeptides were extracted from aleurone layers incubated without hormone, with 25 µM ABA or 25 µM PA and pulse-labelled with [^{35}S]methionine during the last 6 h of a 16 h incubation. Comparison of the heat treated *in vivo* and *in vitro* polypeptides of ABA-incubated and PA-incubated layers showed that all the *in vitro* polypeptides, with the exception of an $M_r \sim$ 31,000 polypeptide, matched with a co-migrating *in vivo* polypeptide marked by (+). An $M_r \sim$ 30,000 polypeptide detected *in vivo* (marked *) was previously shown to be secreted (Fig. 1.8). A precursor-product relationship may be suggested for these polypeptides (*in vitro* $M_r \sim$ 31,000 and *in vivo* $M_r \sim$ 30,000) which have a small difference in M_r. The ABA- and PA-promoted mRNAs programmed the synthesis of *in vitro* polypeptides of $M_r \sim$ 100,000, 68,000, 48,000, 44,000, 34,000, 25,000, 21,000, 20,000, 19,000 and 15,000. However, there were several

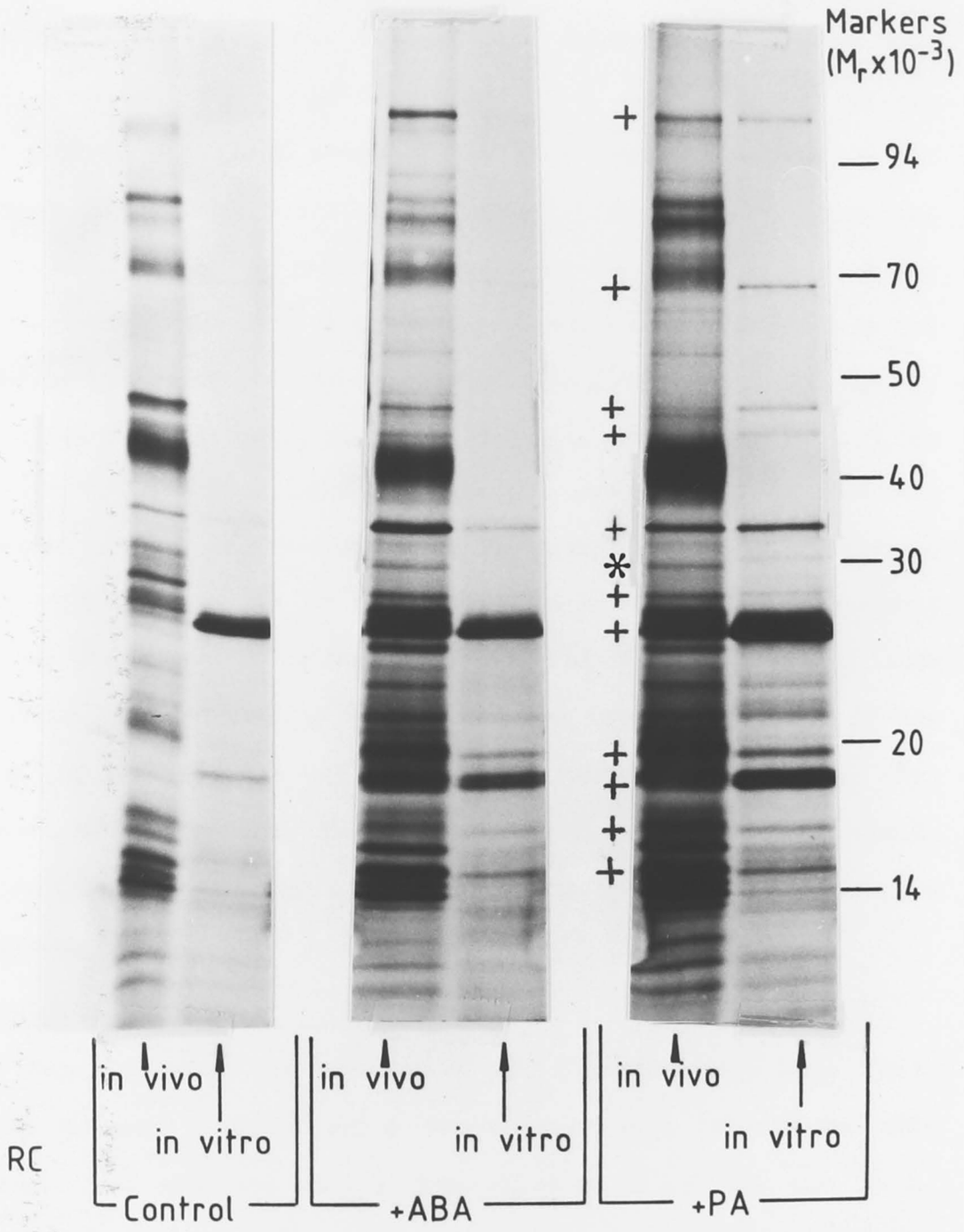
Figure 2.5 Fluorogram of an SDS-polyacrylamide gel.

Comparison of *in vivo* pulse-labelled polypeptides with *in vitro* translation products for Control (no hormone), ABA and PA treatment.

For *in vivo* pulse-labelled products: Aleurone layers were incubated without hormone, with 25 µM ABA or 25 µM PA for 16 h and pulse-labelled with [^{35}S]methionine during the last 6 h. Proteins were extracted, heated at 70°C for 10 min and prepared for electrophoresis. Each *'in vivo'* track was loaded for 0.1 aleurone equivalents.

For *in vitro* translation products: Aleurone layers were incubated without hormone, with 25 µM ABA or 25 µM PA for 16 h. Total RNA was isolated, and translated in a reticulocyte cell-free translation system in the presence of [^{35}S]methionine. 200,000 of TCA-precipitable cpm of each reaction was heated at 70°C for 10 min and prepared for electrophoresis as in Methods.

RC: reticulocyte products with no added RNA.


Markers
(M_rx10^{-3})
94
70
50
40
30
20
14
RC
in vivo
in vitro
Control
in vivo
in vitro
+ABA
in vivo
in vitro
+PA

in vivo polypeptides, particularly the abundant group of polypeptides at $M_r \sim 40,000$ which did not co-migrate with any *in vitro* polypeptides.

For the Control layers, profiles for the *in vivo* and *in vitro* proteins which had been heat treated were different. There was some residual α-amylase ($M_r \sim 46,000$) present after heat treatment of the *in vivo* protein and there seemed to be some degree of induction for ABA-induced polypeptides, particularly the $M_r \sim 40,000$ polypeptide (see Discussion). Likewise, as previously noted in Fig. 2.4 (prints of the same track) the abundant $M_r \sim 25,000$ polypeptide was translated in the reticulocyte system from mRNA of control aleurone layers although it was shown (in Fig. 2.1 using the wheat germ system) that the $M_r \sim 25,000$ polypeptide represented a product from an ABA-promoted mRNA. The presence of promoted levels of ABA-induced polypeptides and mRNAs in Control aleurone layers may be due to the dehydration of aleurone layers during the period of hydration (4-day imbibition) of the grains or at some stage during the isolation of aleurone layers (see Discussion). In the RC track, in which no RNA was added to the translation reaction, heat treatement of the ***in*** *vitro* translation products resulted in virtually total precipitation of the reticulocyte translation products encoded by endogenous mRNAs.

Construction and Screening of cDNA Clones

Dr Peter Chandler had constructed cDNA (complementary DNA) clones using the plasmid pBR322 as a vector and these represented mRNA populations from aleurone layers treated with 25 μM ABA for 16 h. Recombinant clones were initially screened by ampicillin-sensitivity and tetracycline-resistance.

Further screening included colony hybridization and Northern analyses. For colony hybridization, bacterial cells from a culture were spotted onto nitrocellulose filters, treated to lyse the cells and the DNA baked onto the filters at 80°C under vacuum. Duplicate filters were hybridized to radioactive cDNAs made to poly(A)$^+$ RNAs isolated from [-ABA] (incubated for 16 h without ABA) and [+ABA] (incubated for 16 h with 25 μM ABA) aleurone layers.

The purpose of colony hybridization was to look at the differential hybridization of a plasmid to the [-ABA]cDNA and the [+ABA]cDNA probes which should contain more of the ABA-promoted mRNA sequences. In Fig. 2.6, the recombinant clones on the colony filters exhibited three patterns of hybridization to [-ABA]cDNA and [+ABA]cDNA probes:

i) higher level of hybridization to [+ABA]cDNA. These were 'ABA responders'. For example, compare hybridization intensity of colony at co-ordinates A2 in Fig. 2.6a and 2.6b.

ii) higher level of hybridization to [-ABA]cDNA, the 'ABA downers', for example, colony at co-ordinates C9 in Fig. 2.6a and Fig. 2.6b.

iii) equal hybridization to [-ABA] and [+ABA]cDNAs, the 'ABA constants', for example, colony at co-ordinates G6, in Fig. 2.6a and Fig. 2.6b.

Clones classified as 'ABA' downers' may carry sequences induced by GA_3 since these two growth regulators frequently act in opposition. In hybridization experiments, many of these clones have been shown to hybridize strongly to α-amylase cDNA clones (pHV 19: high pI family and clone E: low pI family of α-amylase).

Restriction enzyme (Pst I) digestion on clones classified as 'ABA responders' revealed insert lengths of 190 base pairs (bp) to 600 bp, see Table 1.

Figure 2.6 Autoradiograms of duplicate colony filters hybridized to cDNA probes, showing differential hybridization of clones to (6a) (-ABA)cDNA and (6b) (+ABA)cDNA.

Colour key for colony types:

orange: 'ABA responders'

red: 'ABA downers'

green: 'ABA constants'.

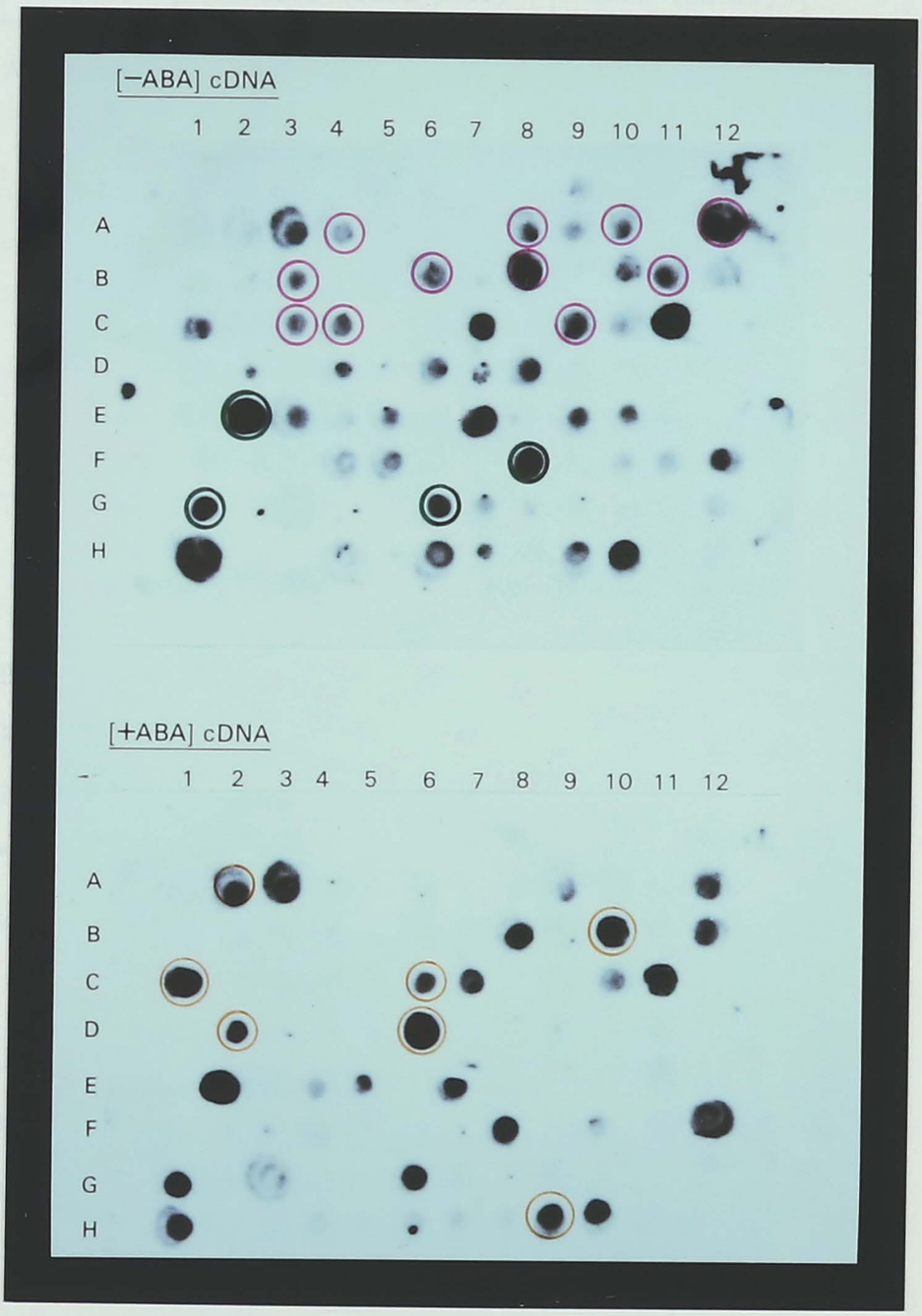

a
[−ABA] cDNA
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
b
[+ABA] cDNA
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

TABLE 1

ABA-induced cDNA clone	Size of Insert (base pair)	Size of mRNA (bases)
pHV A39	200	980+ 1200*
3	300	680+
31	600	660+
35	190	520+
34	500	460+ 580*
24	430	380+
6	300	360+

Footnote

* Sizes of mRNAs were determined by using size markers of mRNAs corresponding to pPS 15-24 (1600 nucleotides) and pPS 15-91 (660 nucleotides). These cDNA clones were previously described by Chandler *et al.* (1984).

+ Size of mRNAs determined from a calibration curve using 18S (1850 nucleotides) and 25S (3700 nucleotides) mRNAs as size markers.

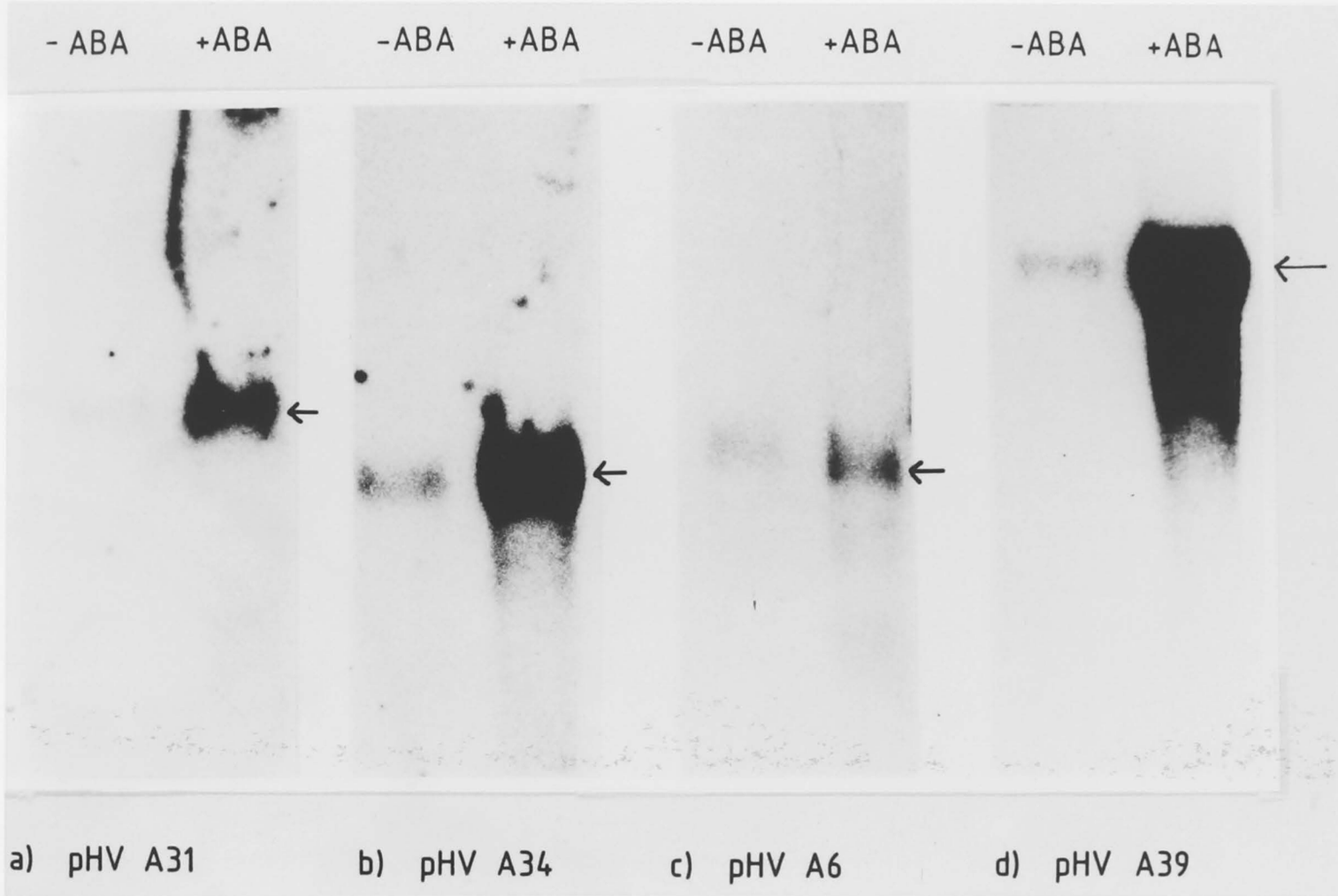
Northern Hybridization Studies

ABA-Induced Changes in mRNA Abundance

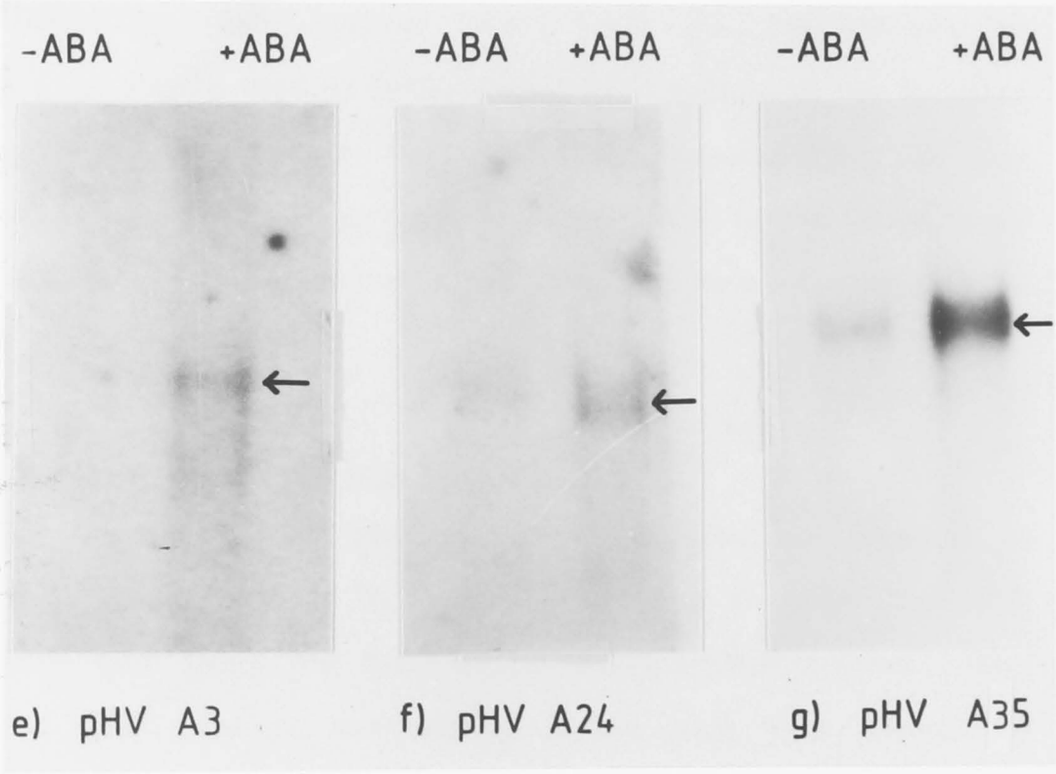
Northern hybridizations were carried out to obtain the size and abundance of the mRNA corresponding to a particular clone. Total RNAs were isolated from aleurone layers incubated without ABA [-ABA] or with 25 μM ABA [+ABA] for 16 h. Samples of total RNA (10 μg) were denatured, electrophoresed on a MOPS-formaldehyde agarose (denaturing) gel and transferred to Hybond-nylon filters. Probes were prepared from inserts of cDNA clones labelled by nick-translation and hybridized to the filters. Fig. 2.7 shows autoradiographs of filters hybridized to radioactive probes made from clones pHV A31, A34, A6, A39, A3, A24 and A35. [-ABA] and [+ABA] total RNAs hybridized differentially to the ABA-induced cDNA probes because [+ABA] total RNA contained induced levels of particular mRNAs, as already shown by cell-free translation data (Figs. 2.1 and 2.4), which assayed for translatable mRNAs: in contrast Northern hybridization analyses measure the relative levels of total mRNAs through detection of homologous sequences using radioactive probes. Positions of mRNA bands which were more intense in the [+ABA] tracks were marked with arrows. For each set of [-ABA] and [+ABA] total RNA the relative intensity of hybridization indicated the relative levels of a specific mRNA in untreated and ABA-treated aleurone layers.

Northern analyses indicated that ABA promoted specific mRNAs to different levels of abundance. The mRNAs represented by pHV A34 and pHV A39 exhibited the highest levels of hybridization to [+ABA] total RNA. Using densitometry, see Fig. 2.8, it was calculated that during

Figure 2.7(a-d) Northern blots showing hybridization of (-ABA) and (+ABA) total RNA to ABA-induced cDNA clones. Preparation of Northerns: 10 µg of total RNA isolated from aleurone layers incubated without (-ABA) and with 25 µM ABA(+ABA) were electrophoresced on formaldehyde gels, blotted onto nylon filters and probed with labelled DNA of inserts obtained from (a) pHV A31, (b) pHV A34, (c) pHV A6, (d) pHV A39 (e) pHV A3, (f) pHV A24 and (g) pHV A35. The filters were then autogradiographed.


- ABA
+ABA
-ABA
+ABA
-ABA
+ABA
-ABA
+ABA
a) pHV A31
b) pHV A34
c) pHV A6
d) pHV A39

esced
and
m


-ABA
+ABA
-ABA
+ABA
-ABA
+ABA
e) pHV A3
f) pHV A24
g) pHV A35

a 16 h incubation of aleurone layers in 25 μM ABA, the message represented by pHV A34 was induced approximately 19-fold, while the message represented by pHV A39 was increased by 25-fold.

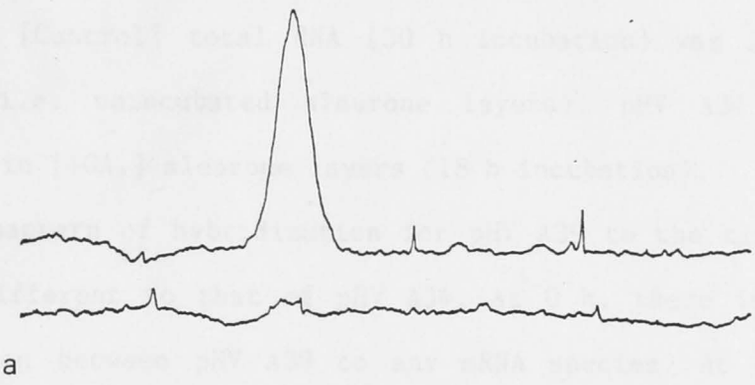
Estimates of the size of mRNAs represented by the cDNA clones were listed in Table 1. These estimates (see footnote) were made from calibration curves based on ribosomal RNA size markers (28S, 3700 nucleotides; 18S, 18500 nucleotides) which were electrophoresed in the lanes adjacent to either the [-ABA] or [+ABA] total RNA. The 28S and 18S ribosomal RNAs were probed by subsequent hybridization of the Northern blots to radioactive cDNA made to 18S and 28S ribosomal RNAs. The ABA-induced mRNAs, which ranged in size from 360 bases to 980 bases, have coding capacities for fairly small proteins with molecular weights of 12,000 to 33,000 daltons.

Northern Analysis for Time-Course RNA

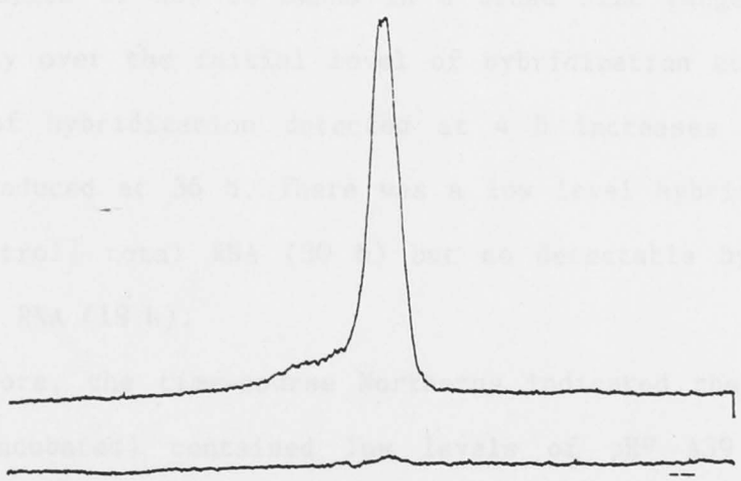
The time-course for ABA-induced polypeptides as detected by pulse-labelling was shown in Fig. 1.2. The Northern analyses (Fig. 2.7) demonstrated that there was differential hybridization between [-ABA] and [+ABA] total RNA to radioactive probes made from inserts of ABA-induced cDNA clones, and provided evidence that ABA induced changes in the levels of several different mRNAs. Since there was variation in the time-course for synthesis and decline of synthesis of the 'ABA polypeptides' it was possible that three may also be variation in the time of induction for the ABA-induced mRNAs.

Total RNAs were isolated from aleurone layers incubated in 25 μM ABA for 0 h, 4 h, 12 h, 24 h and 36 h; without hormone for 30 h or with 1 μM GA_3 for 18 h. Figure 2.9 shows time-course Nothern blots which

Figure 2.8 Microdensitometry plots for Northerns of pHV A34 and pHV A39. Densitometry, using a Joyce-Loebel Mk II microdensitometer was performed on autoradiographs of Northerns probed with (a) pHV A34 (shown in Fig. 2.7b) and (b) pHV A39 (shown in Fig. 2.7d). The baseline was obtained by starting the densitometry plot to one side of the hybridization bands.



a



b

were hybridized to the pHV A34 and pHV A39 inserts. The level of hybridization of A34 insert to a mRNA species is comparatively low at 0 h. When incubated in the presence of 25 µM ABA, the intensity of hybridization increased for up to 24 h, after which the amount of hybridizaiton was reduced at 36 h.

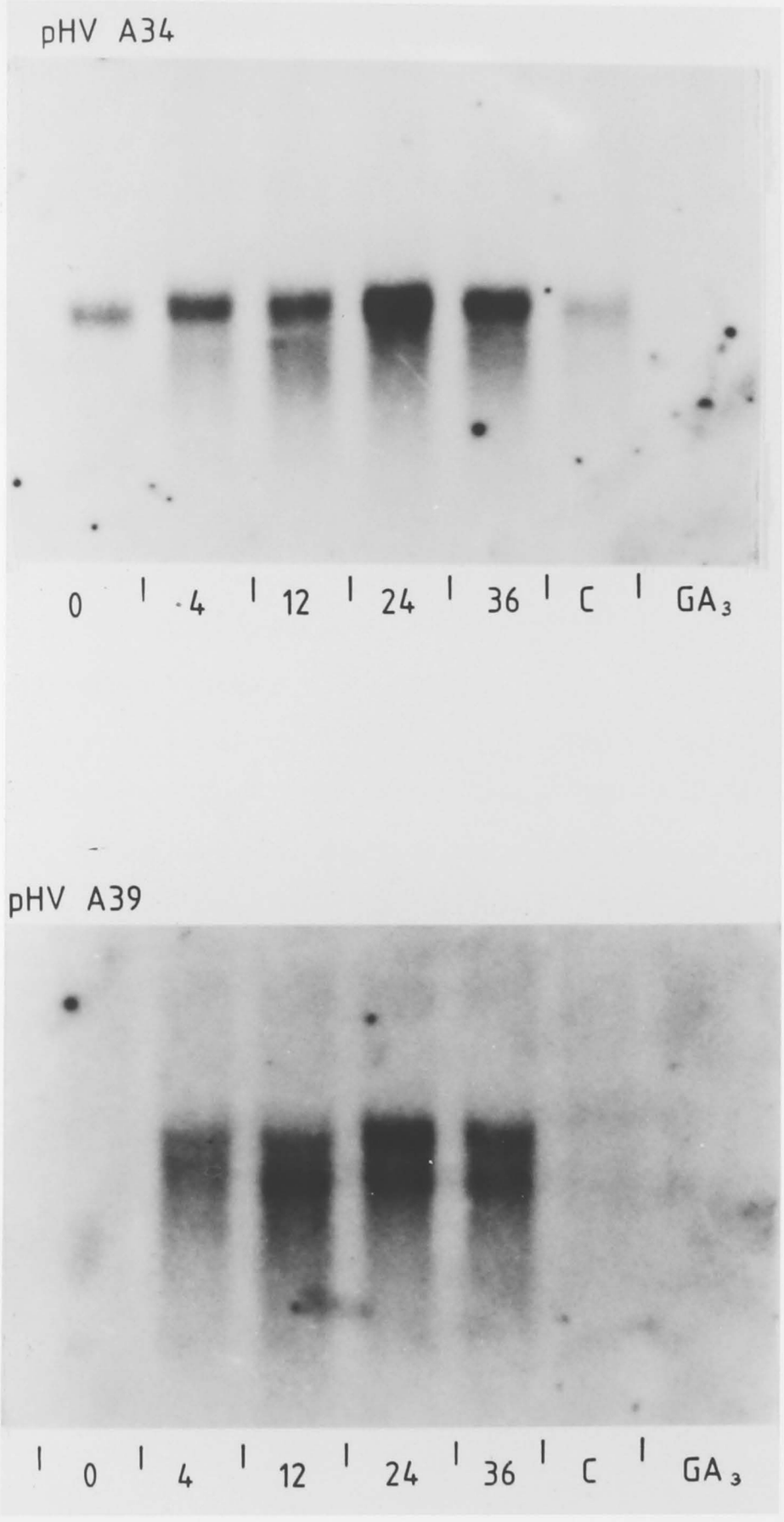
The level of hybridization between A34 and its corresponding message in [Control] total RNA (30 h incubation) was lower than that at 0 h (i.e. unincubated aleurone layers). pHV A34 mRNA was not detectable in [+GA_3] aleurone layers (18 h incubation).

The pattern of hybridization for pHV A39 to the time-course total RNAs was different to that of pHV A34. At 0 h, there is no detectable hybridization between pHV A39 to any mRNA species. At 4 h, the level of hybridization of A39 to mRNAs in a broad size range had increased significantly over the initial level of hybridization at 0 h. However, the level of hybridization detected at 4 h increases slightly by 24 h and was reduced at 36 h. There was a low level hybridization of pHV A39 to [Control] total RNA (30 h) but no detectable hybridization to [+GA_3] total RNA (18 h).

Therefore, the time-course Northerns indicated that 0 h aleurone layers (unincubated) contained low levels of pHV A39 mRNA, but no detectable level of pHV A39 mRNA. In the presence of ABA these two species of mRNA displayed increased abundance by 4 h and reached maximal levels at about 24 h. The presence of GA_3 resulted in suppressed levels of both mRNAs.

As shown above, pHV A34 hybridized to a mRNA species which was represented by a single band. In contrast, pHV A39 hybridized to mRNA species represented by two bands which indicated that the two mRNAs share some sequence homology.

Figure 2.9 Northerns of time-course RNAs probed with (a) pHV A34 and (b) pHV A39. Total RNAs were isolated from aleurone layers incubated in 25 µM ABA for 0 h to 36 h, without ABA for 30 h (control) and with 1 µM GA_3 for 18 h. Northern blots (10 µg total RNA per track) were prepared in duplicate and probed with labelled insert DNA obtained from pHV A34 and pHV A39.


pHV A34
0
4
12
24
36
C
GA3
pHV A39
0
4
12
24
36
C
GA3

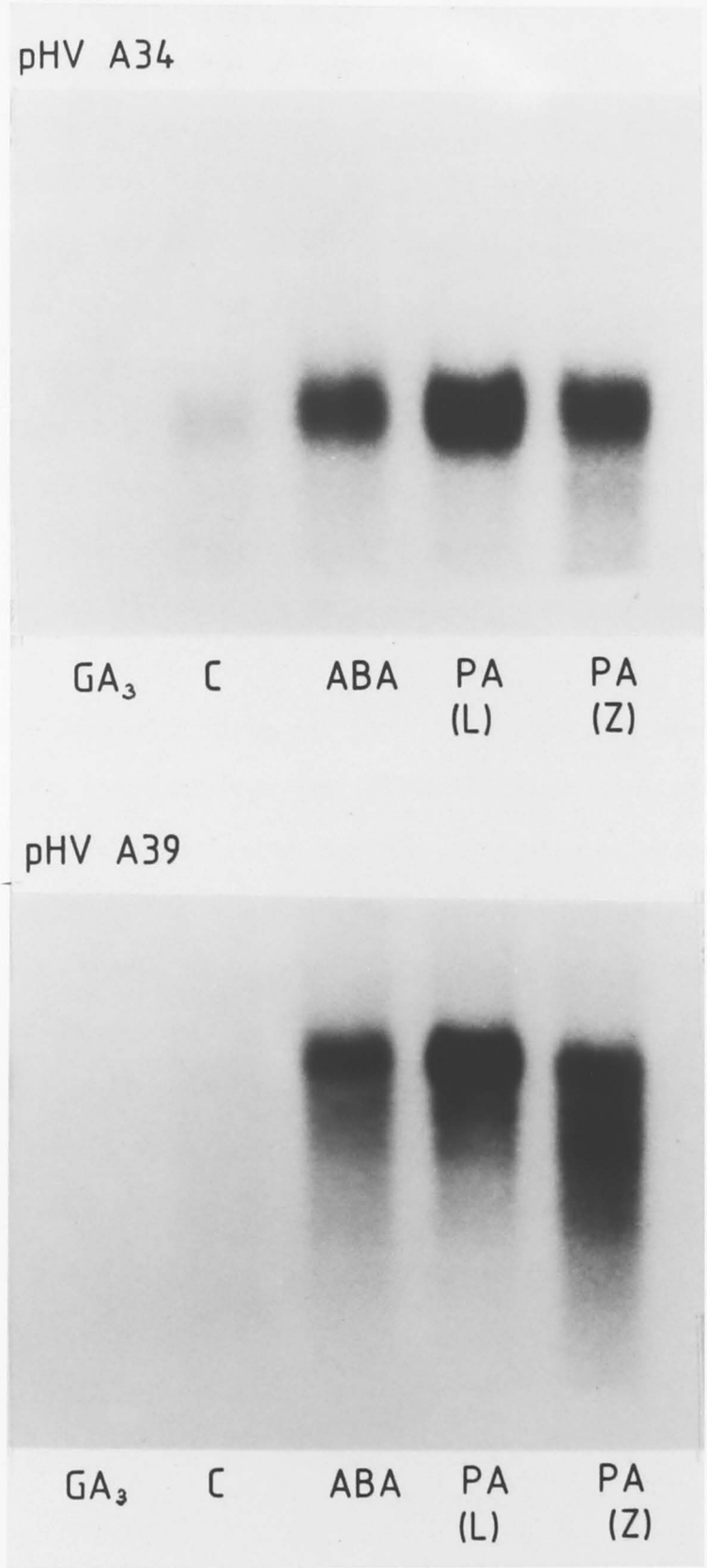
Northern Analyses for PA-treated Aleurone Layers

It was shown in Fig. 1.5 that PA is as active as ABA in inducing the synthesis of the 'ABA polypeptides', and that the changes that can be observed at the protein level were probably due to the effect of ABA on the abundance of certain mRNA species, based on in vitro translation studies (see Fig. 2.7). At present, there is no direct demonstration of the effect of PA on mRNA abundance. To examine this, hybridization analyses were carried out on total RNAs isolated from aleurone layers incubated with ABA and PA. Since PA is not available commercially, samples of phaseic acid have been obtained from two sources: J.A.D. Zeevaart (Michigan State University, U.S.A.) and B. Loveys (CSIRO Division of Horticultural Research, Australia), and the PA samples are denoted PA(Z) and PA(L) respectively.

The hybridization analysis is shown in Fig. 2.10. Total RNAs were isolated from aleurone layers incubated for 16 h without hormone; with 25 µM ABA or 25 µM PA obtained from either Loveys (PA(L)) or Zeevaart (PA(Z)P; and 1 µM GA_3. Duplicate Northern blots (10 µg total RNA per track) were prepared and hybridized to radioactive probes of pHV A34 and pHV A39 (as shown in Fig. 2.10).

Hybridization of the pHV A34 probe to [+ABA] and [+PA] total RNA resulted in a hybridization band at the same position, which suggests that PA and ABA promoted mRNA species of the same size. The intensity of the hybridization band for the [ABA] and [PA(Z)] tracks were approximately equal, however the intensity of the hybridization band for the [PA(L)] track was greater. A possible explanation for this was that [PA(L)] total RNA was isolated in a different batch of RNA preparation where ABA-induced levels of mRNA may be different. The pHV

Figure 2.10 Northerns on the comparison of mRNAs induced by ABA and PA. Total RNAs were isolated from aleurone layers incubated for 16 h without addition (control), in 25 µM ABA, 25 µM PA (obtained from Loveys, PA(L) and Zeevaart, PA(Z) and 1 µM GA_3 (18 h incubation). Northern blots (10 µg total RNA per track) were prepared in duplicate and probed with labelled insert DNA from pHV A34 and pHV A39.


pHV A34
GA3
C
ABA
PA (L)
PA (Z)
pHV A39
GA3
C
ABA
PA (L)
PA (Z)

A34 mRNA was not detected in [+GA_3] total RNA, but was detectable at a low level in [Control] total RNA, as was observed in Fig. 2.9.

The level of hybridization of pHV A39 to [+ABA] and [+PA] total RNA was increased many-fold over that of the [Control] total RNA. The level of hybridization of the pHV A39 probe to total RNA in the PA(L) track was greater than the level of hybridization observed in the [+ABA] and [+PA(Z)] tracks, as was observed for pHV A34, and the explanation offered above is also applicable. Some disperse non-specific hybridization (that appeared as a smear) was observed particularly for the [PA(Z)] total RNA. pHV A39 mRNA was not detectable in [Control] and [+GA] total RNAs. Therefore, these hybridization data displayed the induction by PA of the same species of mRNA to approximately the same steady-state levels, as ABA.

The duplicate Northern blots of Fig. 2.10 also contained tracks loaded for total RNA isolated from pea (*Pisum sativum*) cotyledons. Pea cotyledon cDNA clones are available for the accurate determination of size markers (Chandler *et al.*, 1984). The cDNA clones, pPS 15-24 and pPS 15-91 hybridized to mRNA species fo 1600 nucleotides and 660 nucleotides respectively. These smaller size markers were more suitable for constructing a calibration curve to determine the sizes of the pHV A34 and pHV A39 mRNAs which were more accurately estimated to be 580 and 1200 nucleotides, respectively (see Table 1). Ribosomal markers were previously used to determine the sizes of mRNAs for all seven cDNA clones (see Table 1).

Northern Analysis for Aleurone Layers from Developing Grains

Goldbach and Michael (1976) showed that during barley grain development, the levels of endogenous ABA increased prior to maximum fresh weight. The maximum level of endogenous ABA was attained at about 30 days after anthesis, when rapid growth and accumulation of storage reserves in the endosperm was over.

It was not known if developing aleurone layers were responsive to the increasing endogenous ABA levels, indicated by changes in specific mRNAs. However, since the completion of this study, Mundy *et al.* (1986) have made some investigations on the mRNA abundance of an ABA-induced polypeptide, ASI (α-amylase/subtilisin inhibitor) in the developing barley aleurone layer, by *in vitro* translation and immunoprecipitation. ASI polypeptide was not detected amongst the *in vitro* translation products of developing aleurone RNA.

In preparation for investigations on the responsiveness of developing aleurone layers to endogenous ABA, barley plants were grown in the phytotron (growth conditions in Methods) and the grain fresh weights were determined at various times (number of days) after awn emergence (DAE). The maximum fresh weight was attained at 50 DAE, after which the fresh weight was found to decrease (at 60 DAE). On the basis of the data of Goldbach and Michael (1976), the endogenous ABA level was expected to increase during the period of 20 to 50 DAE, therefore total RNAs were isolated from grains at these stages of development. There were difficulties with isolating aleurone layers from 60 DAE grains because the endosperm had become hard through loss of water.

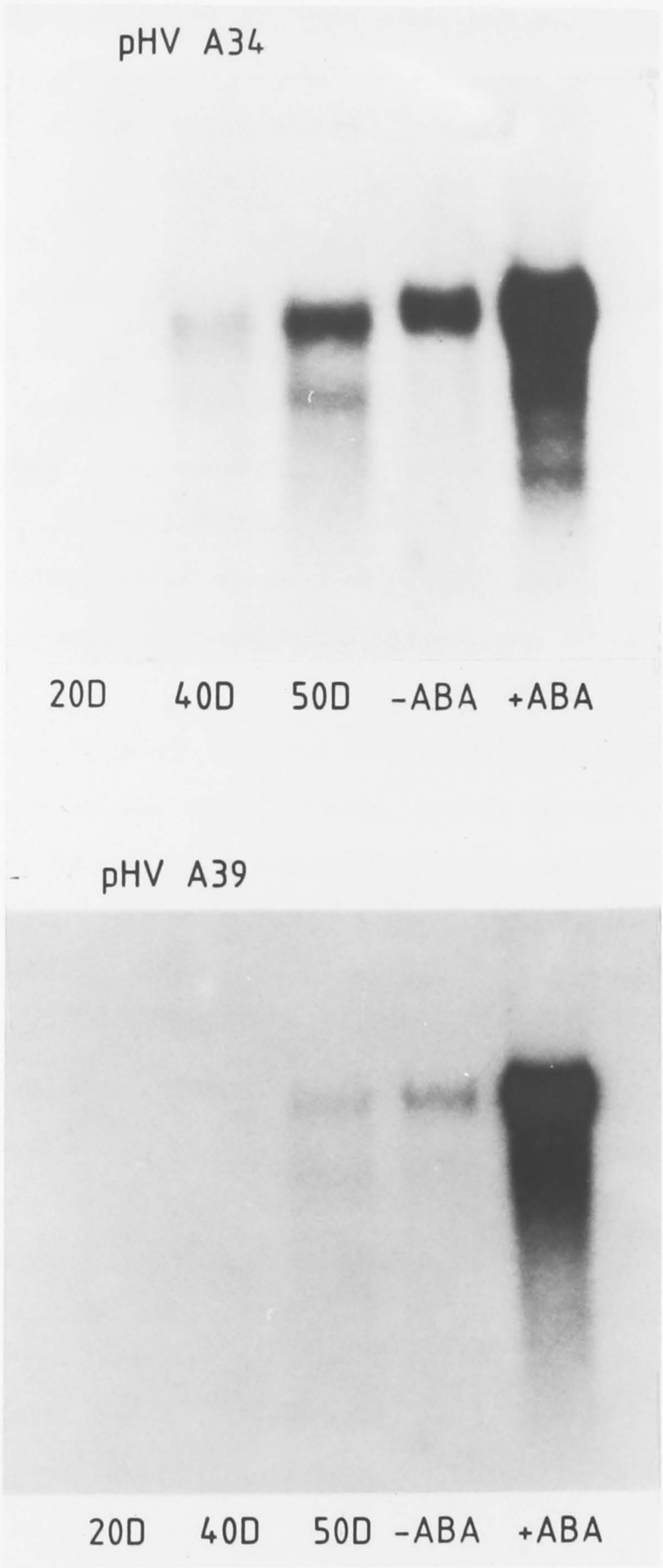
Figure 2.11 shows the result of hybridization of the pHV A34 and pHV A39 probes to total RNAs isolated from developing barley grains at 20, 40 and 50 DAE.

Total RNA isolated from developing grains exhibited no detectable hybridization to the pHV A34 probe until 40 DAE, when there was a low level of hybridization. At 50 DAE, the level of hybridization had increased by several-fold and pHV A34 also hybridized (at a low level) to another smaller mRNA species. By comparison, the level of hybridization of A34 to [-ABA] total RNA from mature 'germinating' aleurone layer was higher, and much higher still following incubation in ABA[+ABA].

When pHV A39 was used as a probe, hybridization was not detectable in total RNA from developing aleurone, until 50 DAE, when there was low level hybridization to an mRNA species corresponding to the pHV A39 cDNA probe, and also to another smaller species which may contain closely-related sequences. The level of hybridization of pHV A39 to [-ABA] total RNA was higher than the maximum level of hybridization detectable for total RNA isolated from developing aleurone layers.

The hybridization patterns for the developmental Northerns demonstrated changes in abundance of specific aleurone mRNAs, perhaps in response to the increase of endogenous ABA during grain development. There was a difference in the time of appearance for mRNAs corresponding to pHV A34 and pHV A39. The earlier induction of pHV A34 mRNA suggested that it was induced at a lower concentration of endogenous ABA than that required for the induction of pHV A39 mRNA. Since levels of hybridization at 50 DAE were lower than those for [-ABA] mature aleurone,

Figure 2.11 Northerns of RNA from developing aleurone layers. Barley plants were grown in a phytotron under 18°C/13°C day-night temperatures. Awns were harvested at 20 days, 40 days and 50 days after emergence. Aleurone layers were isolated from the endosperm by peeling off with a surgical blade, frozen over liquid nitrogen vapour and thawed prior to isolation of RNA. Duplicate Northern blots were prepared by loading for 10 µg of total RNA from the developing aleurone series and mature aleurone layers incubated for 16 h without (-ABA) and with ABA (+ABA), and probed with labelled insert DNA from pHV A34 and pHV A39.


pHV A34
20D 40D 50D -ABA +ABA
pHV A39
20D 40D 50D -ABA +ABA

it was possible that promotion of these mRNAs continued after 50 DAE to reach higher levels carried through to maturity, as reflected by the level of mRNAs of [-ABA] mature aleurone.

Sequence of pHV A34

The insert of pHV A34 (Pst I fragment, approximately 500 bp) was subcloned into the Pst I site of the plasmid pUC 19, and the restriction map determined as shown in Figure 2.12. The nucleotide sequence of pHV A34 was obtained for both strands of the cDNA clone, with sufficient overlaps of nucleotide sequence obtained from selected restriction sites that had been end-labelled as shown in Fig. 2.12. Arrows indicate the sequencing strategy used, 5' end-labelling above, and 3' end-labelling below the diagram.

The nucleotide sequence on each strand was translated into an amino acid sequence in all three possible reading frames by using a computer programme. Out of the 6 possibilities, one open reading frame was selected as the most likely and this is shown in Fig. 2.13.

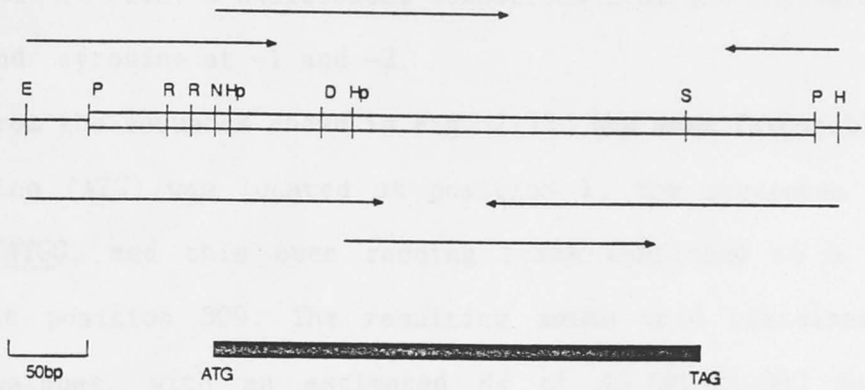
Criteria used for selection of the most probable open reading frame are as follows:

1) The longest sequence available which starts at a methionine residue (ATG) and ends with a STOP (TGA, TAG or TAA) codon.

2) The most favoured initiator methionine residues have the following consensus sequence for nucleotides flanking the initiator codon: (Kozak, 1981; Kozak, 1986)

$$\begin{matrix} \frac{G}{A}CC\ \underline{ATG}\ G \\ -3 \qquad +4 \end{matrix}$$

Figure 2.12 Restriction map of pHV A34 (460 bp) PstI fragment (indicated by double line) sub-cloned into pUC 19 (indicated by single line). Sequencing strategy is indicated by arrows: 5' kinase labelled sites above, and 3' fill-in labelled sites below diagrams. Nucleotide sequence data obtained from each site stretches from the base to the head of arrow.
Key for restriction enzyme sites:
E = EcoR I, P = Pst I, R = Rsa I, N = Nco I,
Hp = Hpa II, D = Dde I, S = Sau 3a and H = Hind III.
The coding region within pHV A34 is indicated by region spanning from ATG to TAG.


E
P
R
R
N
Hp
D
Hp
S
P
H
50bp
ATG
TAG

i.e. the sequence is expected to have a purine in position

-3 (i.e. 3 nucleotides upstream from the initiator codon)

and +4 (i.e. 4 nucleotides downstream from the initiator codon)

and cytosine at -1 and -2.

From the sequence shown in Fig. 2.13, the most favourable initiator methionine (ATG) was located at position 1, the sequences flanking it are GCCATGG, and this open reading frame continued to a STOP codon, (TAG) at position 309. The resulting amino acid contained 102 amino acid residues, with an estimated MW of 10,000-11,000 daltons. This estimate results in the expectation that the mRNA corresponding to clone pHV A34, has the capacity to encode a small protein of MW 10,000-11,000 daltons. This is consistent with the size estimate for the mRNA of approximately 580 nucleotides. The mRNA size is little more than the length of the insert (457 base pairs) and the coding region is indicated in Fig. 2.12.

The amino acid sequence translated in the open reading frame was compared with amino acid sequences of known proteins compiled in the Dayhoff Protein Bank and no significant homology was found. Therefore, no function could be ascribed to the protein encoded by pHV A34 mRNA.

As mentioned above, there were six possible reading frames in which amino acid sequences could be deduced from the nucleotide sequence of pHV A34 and the most likely reading frame (Fig. 2.13) was selected using several criteria. Four of the other reading frames were eliminated on several grounds: interruption of the amino acid sequence by numerous STOP codons, the absence of initiator methionine residues, or short amino acid sequences, spanning between the initiator methionine residue and a STOP codon.

Figure 2.13 Sequence of pHV A34 and deduced amino acid sequence from a selected open reading frame, continuous for 308 nucleotides. Initiator methionine, ATG, is at nucleotide 1, and the STOP codon is at nucleotide 309.

```
 TAT TAC ATT CCC TGA CAG AGG CAG CGA GTG
 Tyr Tyr Ile Pro *** Gln Arg Gln Arg Val

 GGT GGG TAC ATG GTA ATT AAG ATA CTA GTA
 Gly Gly Tyr Met Val Ile Lys Ile Leu Val

                  -3   1  +4              15
 CGG CTT GTC GGC GCC ATG GAC GGG CCG GGG
 Arg Leu Val Gly Ala Met Asp Gly Pro Gly

 16                                       45
 ATG GAG CTC GCG CGC GGG GCG TCT GCC CTA
 Met Glu Leu Ala Arg Gly Ala Ser Ala Leu

 46                                       75
 GCA TTC CCT CTG GCA GGT GCA GCG CCT GAG
 Ala Phe Pro Leu Ala Gly Ala Ala Pro Glu

 76                                      105
 GTG GCA GTA GCC GGC CGT CCA GTG CGC GCC
 Val Ala Val Ala Gly Arg Pro Val Arg Ala

106                                      135
 GTG CTC GCT GAC GCA ATT CCT GGC GCA GCT
 Val Leu Ala Asp Ala Ile Pro Gly Ala Ala

136                                      165
 CTT GTC CGA GAA GCA CGG GAA ATG GTG GTG
 Leu Val Arg Glu Ala Arg Glu Met Val Val

166                                      195
 CTG GCT CTT CCC CGT GCA AAT CCG CGC CTC
 Leu Ala Leu Pro Arg Ala Asn Pro Arg Leu

196                                      225
 CGC CAC GCC GAC GCC GCC GTC GCC GCC GTC
 Arg His Ala Asp Ala Ala Val Ala Ala Val

226                                      255
 GTA GGA GGA AAG CAG GAG CAG CAC CAG CAG
 Val Gly Gly Lys Gln Glu Gln His Gln Gln

256                                      285
 CGC CGC GCA CAC GGT CGC CTT CGT CTT GAA
 Arg Arg Ala His Gly Arg Leu Arg Leu Glu

286                                  309
 CTC CAT TGG CTT CTG TGG GTG TAG GTA GTA
 Leu His Trp Leu Leu Trp Val *** Val Val

 GAT AGG TGG ATG AAG CAA AGC TAG AGC TTC
 Asp Arg Trp Met Lys Gln Ser *** Ser Phe

 TGA TCA AGG TGA CCG TGT GTT CTC TGT TGT
 *** Ser Arg *** Pro Cys Val Leu Cys Cys

 TGT TGT T
 Cys Cys
```

There was one other reading frame for which the pHV A34 amino acid sequence was deduced, but it was on the opposite DNA strand to that presented in Fig. 2.13. It was not regarded as a likely reading frame for translation because of the absence of an initiator methionine. However, pHV A34 may not be a full-length cDNA clone i.e. it may lack the 5' region of its corresponding mRNA, which would contain the initiator methionine residue. The amino acid sequence deduced from the second possible reading frame contained at least 136 amino acids. Although the number of amino acid residues present between the start of translation and the start of the cDNA clone was not known, a protein containing 136 amino acids would be well within the coding capacity of pHV A34 mRNA (580 bases).

Primer extension is currently being done to confirm which of the two DNA strands represents the nucleotide sequence from which the amino acid sequence is translated in the aleurone cells and also to obtain further information on the 5' region of pHV A34 mRNA.

DISCUSSION

Effects of ABA on mRNA Levels: Cell-Free Translation Studies

It was shown by cell-free translation (in the wheat germ and rabbit reticulocyte systems) that the ABA-promoted changes in protein synthesis were due to changes in the levels of translatable mRNAs. The polypeptide profile produced after a cell-free translation reaction for total RNA isolated from ABA-treated aleurone layers exhibited predominance of a $M_r \sim 25{,}000$ polypeptide, confirming the work of Higgins *et al.* (1982) and Mundy *et al.* (1986).

Higgins *et al.* (1982) showed that the GA_3-promoted changes in α-amylase synthesis reflected changes in the level of translatable α-amylase mRNAs, which could be due to an increase in the rate of transcription or a change in the rate of turnover of α-amylase mRNA. However, it has now been demonstrated (by run-off transcription studies) that the GA_3-promoted increase in α-amylase mRNA abundance is regulated at a transcriptional level (Jacobsen and Beach, 1985; Zwar and Hooley, 1986).

Mundy *et al.* (1986), showed that ABA promoted the level of the mRNA for one of the ABA-induced proteins (ASI), and suppressed the level of GA_3-promoted α-amylase mRNA. Thus, the antagonistic action of ABA and GA_3 seems to be operational at the level of mRNA abundance.

In vitro translation of time-course RNAs (using the wheat germ cell-free system) did not indicate specific times of induction of mRNAs by ABA, as was shown in Figure 1.2b. Similar results (for time-course *in vitro* translation studies using poly(A)$^+$ RNA) were obtained using the rabbit reticulocyte cell-free translation system (data not shown). The results obtained may not reflect the genuine times of appearance

of ABA-induced mRNAs since the *in vitro* translation products of Control and [+ABA] total RNA (both 30 h samples) did not demonstrate clearly, the differences in the levels of mRNAs in control and ABA-treated aleurone, as previously seen in Fig. 2.1.

Effects of PA on mRNA Levels: Cell-Free Translation Studies

In vitro translation of total RNA isolated from aleurone layers incubated in 25 μM PA indicated that PA induces the same changes in the mRNA profile, as did 25 μM ABA. The results suggested that PA and ABA regulated protein synthesis by the same mechanism, i.e. by affecting the abundance of mRNA populations that encoded the 'ABA polypeptides'.

The *in vitro* products of [+ABA] and [+PA] total RNAs reflected a reduction in the level of translatable mRNA for α-amylase, but an increase in the levels of translatable mRNAs for 'ABA polypeptides', particularly the abundant $M_r \sim 25{,}000$ polypeptide.

In vitro translation data further strengthened *in vivo* pulse-labelling data on the similarity between the activity of PA and ABA, at least at the level of protein synthesis and mRNA abundance. These results contradicted previous observations that PA did not induce the synthesis of the 'ABA polypeptides' (Ho *et al.*, 1985).

ABA is metabolised to PA in aleurone layers (Harrison and Walton, 1975), however there is no evidence to suggest that PA can be converted to ABA. Since changes in mRNA abundance and protein synthesis could be affected by PA alone, then it is likely that PA may be the active component in the aleurone system although this cannot be demonstrated directly unless the metabolism of ABA to PA could be inhibited.

Part of the problem associated with current work on PA is due to the non-availability of PA commercially, which brings forth the question of purity. For this reason the phaseic acid used in these experiments was obtained from two independent sources, B. Loveys (Australia) and J.A.D. Zeevaart (U.S.A.), and is known to be free from contamination by ABA.

Chemical Properties of *In Vitro* Translation Products Programmed by PA- and ABA-Induced mRNAs

Consistent with the observation that some of the ABA- and PA-induced polypeptides remained soluble after heat treatment (70°C for 10 min), it has been shown that some of the *in vitro* products of mRNAs programmed by [+ABA] total RNA (in wheat and reticulocyte cell-free systems) and [+PA] total RNA (in reticulocyte cell-free system) were also retained in solution after being subjected to the same heating regime. Therefore the PA- and ABA-induced polypeptides whether synthesised *in vitro* or *in vivo* have the same chemical property, at least with regard to solubility after heat treatment.

In contrast, virtually all of the *in vitro* translation products programmed by mRNAs isolated from [Control] aleurone layers precipitated when subjected to heat treatment. However, in Fig. 2.4, an abundant $M_r \sim 25{,}000$ polypeptide remained in solution after heat-treatment - it could be that the mRNA for this 'ABA polypeptide' was induced due to some factors that caused an increase in the levels of endogenous ABA in the aleurone layers. Total RNA isolated from dehydrated half-grains has been shown to exhibit increased levels of ABA-induced specific mRNAs, as shown by RNA hybridization studies using pHV A34 and pHV A39 as probes (P.M. Chandler, personal communication). Therefore,

dehydration is one possible factor, since the de-embryonated half-grains may be subjected to a certain degree of water stress during the 4-day period of imbibition of water, or the isolated aleurone layers may have been dehydrated during the 16 h incubation (without ABA).

Comparison of *In Vivo* and *In Vitro* Products

In agreement with the work of Higgins *et al.* (1982), the size of the in vitro product of α-amylase (translated in the wheat germ and rabbit reticulocyte systems) was larger than the *in vivo* product. Higgins *et al.* (1982) showed that a signal peptide could be cleaved by the addition of canine pancreas membranes.

The *in vitro* (wheat germ) and *in vivo* products of [+ABA] aleurone layers were of different M_r's with the exception of polypeptides at $M_r \sim 25,000$ to $M_r \sim 30,000$ which co-migrated, but it was not known whether the co-migrating bands represented the same polypeptides.

Comparisons for *in vitro* (rabbit reticulocyte system) and *in vivo* products for heated proteins revealed, more clearly, (with the exception of one polypeptide) that each *in vitro* band co-migrated with an *in vivo* band for [+ABA] and [+PA] aleurone layers. However, there were several *in vivo* bands which did not match with an *in vitro* band. This was most striking in the case of the abundant $M_r \sim 40,000$ (*in vivo*) polypeptide. There was only one (*in vitro*) product, $M_r \sim 31,000$ which did not have an *in vivo* counterpart.

A precursor-product relationship may be suggested between the M_r 31,000 (*in vitro*) and an $M_r \sim 30,000$ (*in vivo*) polypeptide. By analogy with α-amylase (Higgins *et al.*, 1982), the observed M_r difference

may be due to cleavage of a signal peptide by an enzyme present *in vivo*. This idea was supported by the observation that an $M_r \sim 30,000$ polypeptide was secreted by aleurone layers. In the case of α-amylase, the precursor molecule is modified post-translationally by glycosylation and removal of signal peptide prior to secretion. The above mentioned precursor-product relationship may be investigated by the addition of dog pancreas membranes (containing enzymes for removal of signal peptide) to the cell-free translation reaction.

Induction of Specific mRNAs by ABA as Detected by Northerns Hybridization

Induction of a new set of mRNAs by ABA in aleurone layers enabled the construction of cDNA clones. This fact was further utilised during screening of clones, to select colonies which carried recombinant plasmids containing cDNA constructed from ABA-induced mRNAs. The differential hybridization of lysed colonies to cDNA probes made from [-ABA] and [+ABA] total RNAs highlighted colonies which contained ABA-induced sequences, since cDNA probes made from [+ABA] total RNA contained more of those sequences.

The same rationale applied to Northerns in which total RNA (10 µg) isolated from [-ABA] and [+ABA] aleurone layers was hybridized to probes made from the inserts of the cDNA clones. The greater intensity of hybridization of the [+ABA] total RNA compared with [-ABA] total RNA reflected the steady state levels of mRNAs corresponding to the seven cDNA clones and gave an estimate of the level of induction for the particular mRNA. The highest levels of induction for ABA-induced messages were found to be approximately 20-fold for pHV A34 and 25-fold for pHV A39. The higher level of induction for pHV A39 was partially accounted

for by a virtually non-detectable level of its mRNA in [Control] total RNA whereas the pHV A34 message was present at a significantly higher level in [Control] total RNA. Total RNA isolated from GA_3-incubated aleurone layers did not contain any detectable pHV A34 and pHV A39 mRNAs. This observation extended the work of Higgins *et al.* (1982) and Mundy *et al.*(1986) that GA_3 suppressed ABA-promoted changes in mRNA abundance.

These mRNAs also exhibited tissue-specificity (P.M. Chandler, personal communication). In barley seedlings, the pHV A34 message was detectable predominantly in the aleurone layers, while the pHV A39 message was detectable in barley grains as well as shoots and roots. However, data obtained from time-course Northern indicated that the kinetics of induction of pHV A34 and pHV A39 mRNAs were similar. The mRNAs corresponding to pHV A34 and pHV A39 reached maximal levels at about 24 h after which the steady state levels for both mRNAs decreased.

Induction of Specific mRNAs by PA as Detected by Northerns

Hybridization analyses on total RNA isolated from aleurone layers incubated in PA gave the same results as similar experiments on [+ABA] total RNA. For two mRNA species at least, PA induced specific 'ABA mRNAs' resulting in approximately the same level of induction, as judged by the intensity of hybridization bands on Northerns.

Appearance of Specific mRNAs in Aleurone from Developing Grain

The aleurone layer of developing barley grains exhibited increased levels of mRNAs which corresponded to pHV A34 an pHV A39. Based on the data of Goldbach and Michael (1976) on the content of endogenous ABA levels during grain development in other cultivars of barley other than

Himalaya, and having determined the fresh weight of the grains, it was expected that the endogenous ABA levels would increase from about 20 DAE until about 50-60 DAE. The expected increase in the levels of endogenous ABA was corroborated by the increased abundance of the pHV A34 and pHV A39 messages between 20 to 50 DAE. From the correlative evidence presented, it is not certain if the increases in abundance of these messages reflected the responsiveness of aleurone layers to levels of endogenous ABA during grain development.

The levels of pHV A34 and pHV A39 mRNAs accumulated at 50 DAE appeared to have been carried through to maturity, as indicated by the levels of pHV A34 and pHV A39 mRNAs in mature aleurone layers. This was consistent with previous observations (Chapter 1, Fig. 1.2) that 'ABA polypeptides' were synthesised at low levels in [-ABA] aleurone layers at 2 h, but the 'ABA polypeptides' were no longer synthesised at 48 h, probably due to the metabolism of ABA to DPA. Relative to each other, the level of pHV A34 mRNA was consistently higher than that of pHV A39 in 50 DAE (developing) as well as in mature aleurone layers.

The levels of the ABA-induced mRNAs (corresponding to pHV A34 and pHV A39) at 50 DAE were not as high as their respective levels in [-ABA] aleurone layers. It is possible that the ABA induced mRNA levels may have increased further after 50 DAE. At that stage, dehydration of the grains would effectively result in an increased concentration of ABA within the grain, assuming that the amount of ABA (per grain basis) was constant. The amount of ABA per grain was shown to decline as fresh weight decreased in barley cultivars 'Kristina' and 'Oriol' (Goldbach and Michael, 1976). However, this would not necessarily mean that the concentration of ABA within the aleurone layer (and the tissues

adjacent to it) had not increased since it was also possible that during dehydration, most of the ABA somehow partitioned into the aleurone layer. Since the aleurone makes up a small proportion of the grain, then the effective concentration of ABA within aleurone would increase. This may result in increased levels of specific ABA-induced mRNAs, perhaps reaching the levels seen in mature grains (which had not been treated with ABA).

The aleurone layer of developing grains was a more difficult system to work with compared to that of the mature grain. At 60 DAE, dehydration of the barley grains during the maturation process caused difficulties in isolation of aleurone layers from the endosperm which had become desiccated by that stage. Also, total RNA preparations obtained from developing aleurone layers resulted in very low yields in terms of µg total RNA per aleurone layer, compared with yields obtainable from mature aleurone.

Although the level of pHV A34 mRNA was higher than that of pHV A39 in uninduced tissue, the situation was reversed in ABA-incubated aleurone layers i.e. the level of pHV A39 mRNA was induced to a higher level relative to pHV A34. The difference between induced (in [+ABA] aleurone) and uninduced levels of mRNAs obtained by densitometry resulted in values of about 25-fold increase for pHV A39 and 20-fold for pHV A34.

Mundy *et al.* (1986) showed that in developing barley aleurone layers, ASI (α-amylase/subtilisin inhibitor) was not detectable by *in vitro* translation followed by immunoprecipitation, although the synthesis of ASI was promoted by ABA in mature aleurone layers. However, *in vitro* translation together with immunoprecipitation may not be as sensitive

as Northerns in detecting low levels of ABA-induced RNAs. Also, the two techniques used were different in that *in vitro* translation assayed for translatable mRNAs only, while Northerns were used to measure total mRNAs.

The kinetics of induction of 'ABA polypeptides' and the ABA-induced mRNAs could not be related from the data available so far. *In vivo* pulse-labelling data (Chapter 1, Fig. 1.2b) indicated that individual 'ABA polypeptides' were induced at different times and synthesis may proceed for long or short periods. *In vitro* translation data did not indicate specific times for the induction of the ABA-induced mRNAs as judged from the levels of synthesis of all polypeptides programmed by [+ABA] total RNA. Northern analyses indicated that the levels of pHV A34 and pHV A39 mRNAs reached maximal levels between 24 h and 36 h.

SUMMARY

The polypeptide profile of *in vitro* translation products programmed by [+ABA] total RNA reflect changes in mRNA abundance in aleurone layers treated with 25 M ABA.

In the wheat germ system, some of the *in vitro* translation products of [+ABA] total RNA are retained in solution following heat treatment as are the *in vivo* pulse-labelled ABA-induced polypeptides. The *in vitro* and *in vivo* polypeptides may or may not co-migrate.

In the rabbit reticulocyte system, the polypeptide profiles of *in vitro* translation products programmed by [+ABA] and [+PA] total RNAs reflect changes in the same populations of mRNAs.

Some of these *in vitro* products are also retained in solution after heat treatment, as are some of the *in vivo* PA- and ABA-induced polypeptides.

All *in vitro* products (with the exception of an $M_r \sim 31,000$ polypeptide) of [+ABA] and [+PA] total RNAs co-migrate with an *in vivo* counterpart, however several abundant in vivo polypeptides did not have a co-migrating counterpart. A precursor-product relationship may exist between an $M_r \sim 31,000$ *in vitro* polypeptide with an *in vivo* polypeptide ($M_r \sim 30,000$) which is secreted by aleurone layers.

Seven ABA-induced cDNA clones have been characterised in terms of mRNA size and abundance. mRNAs corresponding to pHV A34 and pHV A39 are induced by 19-fold and 25-fold respectively, in ABA-treated aleurone layers.

Time-course Northern analyses for ABA-incubated layers show that the induction of mRNAs for pHV A34 and pHV A39 reached maximal levels at 24 h.

PA also promotes mRNAs that correspond to pHV A34 and pHV A39, to approximately the same levels as that induced by an equal concentration of ABA.

Aleurone layers of developing barley grains exhibit increases in the levels of mRNAs corresponding to pHV A34 and pHV A39. Since the levels of ABA-specific mRNAs were promoted at the stage of development when endogenous ABA levels have been noted to rise in other cultivars of barley, then this provides circumstantial evidence that the increase in ABA-specific mRNAs occurred in response to the endogenous ABA level. The promoted levels of ABA-specific mRNAs reached at the final stage of development may be carried through to maturity.

Deduced amino acid sequence from an open reading frame of the nucleotide sequence of pHV A34 did not have significant homology with any amino acid sequence available in the Data Bank, thus, there is no clue available as to the function of the protein encoded by the mRNA corresponding to the ABA-induced cDNA clone, pHV A34.

FURTHER DISCUSSION

ABA induces the synthesis of several polypeptides and suppresses the synthesis of GA_3-promoted polypeptides in barley aleurone layers. The induction of 'ABA polypeptides' has a requirement for a threshold concentration of ABA, although each of the polypeptides exhibit different kinetics of induction and was synthesised for different durations in response to ABA.

The changes in protein synthesis are regulated primarily at the level of mRNA abundance as revealed by cell-free translation. This enabled the construction of cDNA clones (by P.M. Chandler) which were subsequently used as probes in Northern analyses for RNA isolated from ABA-treated aleurone layers. Time-course Northern analyses indicated maximal induction of their corresponding mRNAs at about 24 h. Developing aleurone layers may be responsive to the endogenous ABA level (which is known to rise during grain development), as indicated by the increased abundance of pHV A34 and pHV A39 mRNAs.

Phaseic acid induces the same changes in protein synthesis and mRNA abundance as ABA. These effects have also been shown to be modulated at the level of mRNA abundance (shown by Northern analyses and cell-free translation studies). These results together provide strong evidence that PA possesses the same activity as ABA in the induction of 'ABA polypeptides' through promotion of ABA-specific mRNAs. The data presented contradict the work of Ho *et al.* (1985), who showed by *in vivo* pulse-labelling, that PA does not induce the 'ABA polypeptides'. Based on the *in vivo* pulse-labelling data on ABA-GA_3 interaction (Chapter 1), further work would include studies on the effect of much lower concentrations of GA_3 on the induction of specific ABA-induced mRNAs

by ABA or PA. The relative steady-state levels of the ABA-induced mRNAs (to be probed with ABA-induced cDNA clones, pHV A34 and pHV A39) seen in 1 μM ABA would be expected to be suppressed by the simultaneous addition of GA_3, at lower than 0.05 μM. By the same token, α-amylase mRNA level is expected to increase due to its promotion by GA_3 and in order to monitor this, pHV 19, a high pI α-amylase cDNA clone (Chandler *et al.*, 1984) would be used as a probe.

The nucleotide sequence of pHV A34 was translated in a particular open reading frame to deduce the amino acid sequence of the protein encoded by its corresponding mRNA. Primer extension is currently being done to confirm that the correct DNA strand was selected for translation of the amino acid sequence. According to the restriction map of pHV A34 (see Fig. 2.12), a 66 bp DdeI-HpaII fragment serves as a suitable primer. The DdeI-HpaII fragment would be kinase labelled at the DdeI site, hybridized to total RNA (enriched in ABA-specific mRNA) and extended from the HpaII site using its mRNA as template. Apart from confirming the correct DNA strand that is translated, primer extension would also provide data on the length of the 5' untranslated region of pHV A34 mRNA.

Another ABA-induced cDNA clone (pHV A39) is also emerging to be an interesting clone from the point of view of its abundance in ABA-treated aleurone layers as well as in several tissues of dehydrated seedlings of barley, maize and wheat (personal communication, P.M. Chandler). The sequencing of pHV A39 will be carried out and the deduced amino acid sequence compared with sequences of known proteins available in the Data Bank. Homology with the sequence of a known protein may provide some clue as to the function of the protein encoded by the gene represented by pHV A39.

Identification of ABA-induced proteins may lead to further understanding on the action of ABA. The function of one of these 'ABA polypeptides' the α-amylase/subtilisin inhibitor (Mundy, 1984) is not known, although it is possible to speculate that in the barley grain, ABA promotes the synthesis of ASI which would inhibit the activity of α-amylase already accumulated in the grain. When barley seedlings are dehydrated slowly, the levels of pHV A34 and pHV A39 mRNAs increased and subsequent rehydration of the seedlings allows seedling growth to be resumed (P.M. Chandler, personal communication). However, rapid dehydration did not reveal increased levels of pHV A34 and pHV A39 mRNAs and seedlings were unable to resume growth when rehydrated. These observations suggest that during slow dehydration, grains may be able to synthesise ABA which affects the abundance of specific mRNAs that encode proteins with a 'protective' function e.g. ASI may inhibit further hydrolysis of the endosperm by inhibiting α-amylase activity when the grains are dehydrated. Also, other correlative evidence for the induction by ABA of 'stress' proteins is available. For example, the level of a specific mRNA species (homologous to the 5' region of the heat shock protein gene of *Drosophila*) increased in maize seedlings when subjected to heat shock, water stress, ABA or wounding (Heikkila *et al.*, 1984). Moreover, two 'stress' proteins were found to be common to the stress treatments (heat shock, water stress, ABA and wounding) although each type of stress also caused the induction of additional unique proteins. Since there is sufficient evidence on the correlation between stress and increased endogenous ABA levels, it may be hypothesised that when plants are subjected to stress the increased endogenous ABA levels may assist the plant in the adaptation to stress by regulating gene expression which is ultimately manifested as increased synthesis of

specific proteins that may act as enzymes, inhibitors or 'masking' proteins (to suppress expression of particular genes that encode proteins which may be undesirable under stress situations).

At this stage, the ABA-induced cDNA clones have not been matched with the 'ABA polypeptides'. This problem could be resolved by hybrid arrest translation, a technique based on the inability of ribosomes to translate mRNA which is hybridized to cDNA. The arrest of translation of a particular mRNA is indicated by the absence of a polypeptide from the spectrum of in vitro translation products. The foreseeable problem of having a co-migrating polypeptide obscure a 'missing' band could be partially alleviated by heat treatment of the translation products, assuming that the background translation product would precipitate and that the polypeptide of interest would remain in solution. Another technique, related to hybrid arrest translation, could also be used as an alternative. Hybrid release translation is assayed by the cell-free translation of a single hybrid-selected mRNA. The cDNA-mRNA hybrid is fractionated from other mRNA populations and denatured prior to translation.

The long-term aim of the present study is to elucidate the mode of action of ABA. The barley aleurone layer has been used extensively as a model system in the understanding of GA_3 action in the enhancement of α-amylase gene expression, and it is certainly an equally favourable system for the study of ABA action. It is now established that the regulation of α-amylase gene expression by GA_3 and ABA operates primarily at the transcriptional level. However, there is no data available on the site of action for either GA_3 or ABA although measurement of endogenous levels of GA_3 and ABA clearly indicate that these plant hormones do enter aleurone cells. Compartmentation studies show that

the undissociated form of ABA can traverse membranes. The question still remains whether ABA and GA_3 remain in the cytoplasm or enter the nucleus before they exert effects such as those on the transcription of α-amylase gene expression.

If these plant hormones were to be found only in the cytoplasm, then clearly, their action on gene expression would be mediated by other molecules which are activated somehow, probably by the hormone and possibly in conjunction with other molecules prior to entry into the nucleus. Detection of ABA and GA_3 in the nucleus on the other hand, would suggest that these plant hormones themselves could act either as inducers or suppressors, depending on the regulatory sequences which may signal shut-down or turn-on of their respective genes.

Some analogy could be drawn with steroid hormones which must enter their target cells to exert their effects (Stryer, 1981). For example, 17β-estradiol binds to a specific receptor (which becomes activated) in the cytoplasm of uterine cells and this hormone-receptor complex subsequently enters the nucleus where the complex interacts with DNA. The impact of 17β-estradiol is not realised until several hours because its biological effect depends on gene transcription which is ultimately manifested as new protein synthesis.

Currently, there is general agreement on the nature of a DNA sequence motif which is bound by steroid receptors (Yamamoto, 1985). The consensus sequence for the binding of glucocorticoid receptor is 16-nucleotides long and there is also evidence for a more detailed involvement of contact nucleotides in the receptor: DNA interaction. These sequences, (called steroid response elements) have been identified

in the mediation of transcriptional regulation of genes by steroids - binding of the hormone receptor to the response elements enhances transcription.

In conclusion, the mode of action of ABA may be exemplified by the action of steroid hormones in the activation of specific genes. Also, since the effect of ABA in suppressing α-amylase gene expression has been shown to be regulated at the transcriptional level, it is likely that similar control mechanisms would be involved in the regulation of synthesis of the 'ABA polypeptides'. Judging from the number of ABA-promoted polypeptides it would not be surprising if several receptors (which recognise different regulatory sequences) were involved in the action of ABA at its primary site. It is also possible that ABA-specific receptors may vary in different tissues or between plant species so that the resultant impact of ABA on gene regulation could be diversified.

The long-term aim of this study is to understand the mode of action of ABA. Since current work on GA_3 promotion of α-amylase transcription is being done to identify regulatory sequences which are involved in the action of GA_3, similar experiments may assist in the identification of specific sequences which are involved in the action of ABA.

The findings to be made at the molecular level may be used to explain the diverse physiological responses which are elicited by ABA. However, due to the diversity of the nature of these responses a single model may not be sufficient to explain the processes involved in all of them.

Based on current knowledge on the effects of ABA on mRNA abundance and protein synthesis as well as the correlation between endogenous ABA levels and environmental stresses (e.g. dehydration, temperature

extremes) it may be speculated that stress somehow provides a 'signal', detected by certain responsive cells, to increase the synthesis of ABA. ABA is then mobilised to reach its target cell; where it may exert its effect in a similar way to steroid hormones (in animals), resulting in changes in mRNA abundance and protein synthesis.

Since the final result of ABA action needs to comply with the initial 'stress signal' then it is likely that ABA action may also be tissue specific. This could be facilitated by the interaction of ABA with a different receptor, i.e. different ABA-receptor complexes may interact with a membrane to change its permeability or with certain sequences, resulting in transcriptional changes of specific genes. These gene products may have 'protective functions' allowing the plant to adapt to a particular stress.

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